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The main object of the Journal is to bring together work concerned with all the kinds of developmental process to be found in animals at any stage of their life-history. It will include work on embryology (descriptive, experimental, chemical, physiological), on regeneration and healing, on the developmental changes involved in functional adaptation, on growth, ageing, and other aspects of late morphogenesis. It will publish studies of gametes and gametogenesis where the work is not primarily of cytological interest; and studies of developmental genetics and of the physiology of reproduction where these fall more within its scope than within the usual scope of existing specialized journals. Papers on those aspects of protozoology which have a bearing on morphogenesis will be acceptable. Papers concerned with developmental processes in plants may be included if they link up with zoological aspects of morphogenesis.

The papers published will for the most part be accounts of finished pieces of research. Preliminary reports will not be published. Theses and very long papers are unlikely to be accepted at present. Theoretical and review papers may be published from time to time, as space allows. Contributions may be English, French, or German. Contributors wishing to use other languages should consult the Editor.

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Stages in the Development of the Stickleback *Gasterosteus aculeatus* (L.)

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From the Department of Zoology and Comparative Anatomy, University of Oxford

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INTRODUCTION

MUCH good work has been done on the developmental morphology of the teleostean fishes from the works of Ryder (1884), Cunningham (1886), and McIntosh & Prince (1890) onwards. With the advent of experimental embryology the need for tables of development of different species was felt. Meek (1924), Ballantyne (1930), and Oppenheimer (1937) provide some normal tables for the teleosts *Gadus*, *Callichthys*, and *Fundulus* respectively, but much work is still needed on other species which are suitable for experimental work. *Gasterosteus aculeatus*, the three-spined stickleback, has become a laboratory fish because of its small size, easy availability, wide distribution, and very interesting breeding habits. This fish breeds mainly during April and May, but it is possible in the laboratory to bring it into breeding condition during most of the year. The factors controlling the time of reproduction are intensity of light and amount of food. When the author began experimental work on *G. aculeatus* it was soon realized that no complete table of normal development of this fish exists. Kuntz & Radcliffe (1917) described only a few stages, and though Vrat (1949) has given the main stages, he has omitted many important developmental features. A more detailed account of the development of this fish has therefore been prepared.

Since development is a continuous process it cannot be subdivided into well-defined morphological stages. Stages based on age can be misleading because the rate of development is greatly influenced by the temperature and oxygen content of the water. A method of description has been adopted where the continuity of the developmental process is maintained and stages are named according to some important, easily discernible morphological characters.

DEVELOPMENT

The eggs and embryos dealt with in this paper were for the first 3–4 days kept in small Petri dishes, the water being changed 2 to 3 times daily. Afterwards they were transferred to small tubes into which tap water dropped at a steady

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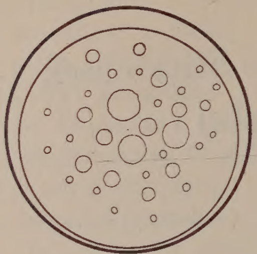

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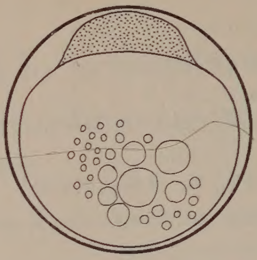

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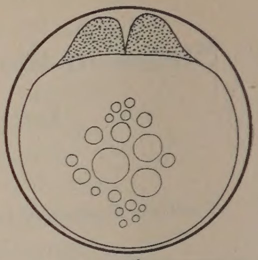

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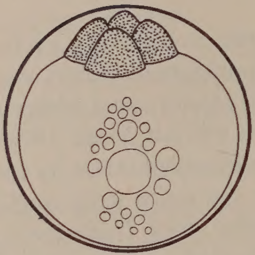

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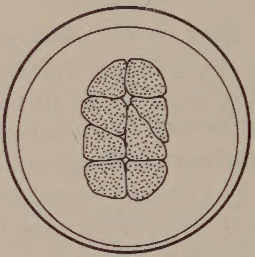

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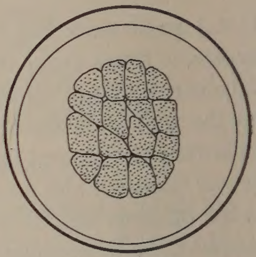

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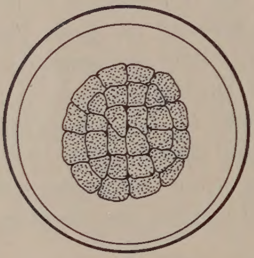

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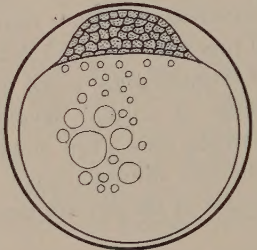

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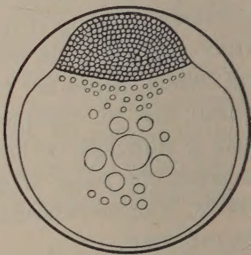

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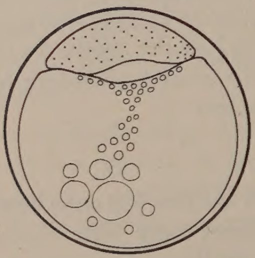

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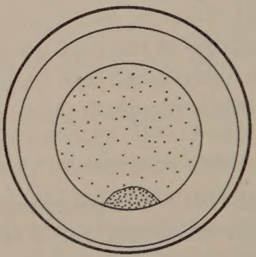

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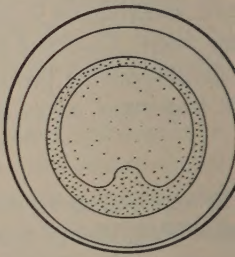

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rate. Here the embryos were kept until the yolk was completely used up. During the whole of this time the temperature remained between 18° and 19° C. Under these conditions the embryos take 6–8 days to hatch, and about 4 more days to complete absorption of the yolk. In another 12 days the young fish will assume all its essential features.

Unfertilized eggs

The female lays about 100 to 150 eggs at a time. The eggs remain together in a mass surrounded by mucus. They are opaque and heavier than water. The eggs of newly caught females are light orange, but if the females have been previously kept in an aquarium and fed on *Tubifex* they are colourless. Soon after oviposition the egg capsule hardens and the eggs become firmly attached to one another.

Each egg is spherical, about 1·2 to 1·7 mm. in diameter. Great differences in size occur not only amongst the eggs of different females but sometimes amongst the eggs of the same female. The outer covering of the egg, known as the shell or the vitelline membrane, is quite thick and tough and possesses a micropyle. The vitelline membrane at this stage is closely applied to the egg proper. The main bulk of the egg is formed of transparent yolk, the cytoplasm proper being an extremely thin peripheral layer round the yolk. The ovum possesses many oil globules, the larger always lying in the yolk. There is no indication of polarity in the unfertilized egg.

Stage 1. Fertilized egg (Fig. 1)

Immediately after fertilization the egg starts to become gradually more and more transparent, the vitelline membrane separates from the egg proper, and the perivitelline space, filled with its fluid, develops.

Stage 2. Blastodermic cap ($1\frac{3}{4}$ hours after fertilization) (Fig. 2)

About 30 minutes after fertilization of the egg, waves of contraction set in at the vegetative pole, pass through the equator, and end at the animal pole, where an accumulation of cytoplasm takes place. The completion of one contraction cycle takes about 2 minutes. About twenty of these cycles follow one another, each cycle adding more and more cytoplasm at the animal pole, which after about $1\frac{3}{4}$ hours forms a cap known as the blastodermic cap or disk. The animal pole can be recognized at the beginning of the first wave of contraction.

Stage 3. 2-cell stage ($2\frac{1}{2}$ hours after fertilization) (Fig. 3)

Segmentation begins about $1\frac{3}{4}$ hours after fertilization. The raised circular blastodermic disk at the animal pole flattens and elongates into an oval shape. A cleavage furrow, at first superficial, appears in the plane of the shorter axis of the disk, which then divides completely into two blastomeres. These begin to bulge out again and give rise to the 2-cell stage.

Stage 4. 4-cell stage (3 hours after fertilization) (Fig. 4)

The flattening is repeated at the 2-cell stage and when it reaches its maximum a second cleavage furrow appears at right angles to the first. By cutting deeper this furrow separates the four blastomeres which bulge out again and give rise to the 4-cell stage.

Stage 5. 8-cell stage (3½ hours after fertilization) (Fig. 5)

The flattening of the blastomeres is repeated. The second cleavage furrow remains straight, but the first now becomes irregular. The four blastomeres elongate in the direction of the second cleavage furrow. Two new cleavage furrows appear on either side of the first and at right angles to the second. Thus eight blastomeres are formed in two rows of four and when these two furrows deepen and the eight blastomeres bulge out, the 8-cell stage is reached. In side view the four central cells stand a little higher than the four terminal cells.

Stage 6. 16-cell stage (3¾ hours after fertilization) (Fig. 6)

After the 8-cell stage the flattening and bulging out of the blastomeres is not so pronounced. Two new vertical furrows become visible. They are parallel to and on either side of the second cleavage plane and at right angles to the first and third. In this way sixteen cells are formed, all of which lie in a single layer.

Stage 7. 32-cell stage (4 hours after fertilization) (Fig. 7)

The 16-cell stage undergoes further division, but from now on the cleavage furrows are horizontal as well as vertical. The four central cells are divided by a horizontal division into eight cells which are arranged in two layers of four cells each. With the exception of the four corner cells in which the division is more or less diagonal, the rest of the cells are divided by vertical divisions. These are either parallel to the first or to the second cleavage furrows. In this way the 32-cell stage is formed.

Stage 8. Early morula (6 hours after fertilization) (Fig. 8)

Beyond the 32-cell stage the cleavage divisions are difficult to follow. Vertical as well as horizontal cleavage furrows are formed and the cells become smaller in size. The total area occupied by the cells of the early morula is more or less the same as that of the original blastodermic disk.

Stage 9. Late morula (10 hours after fertilization) (Fig. 9)

The cells of the morula divide further and become smaller in size. In a side view this stage appears as a mass of cells with a prominent hemispherical projection and a convex base which rests in a shallow concavity of the yolk. A large number of oil globules pass out from the cell mass into the yolk, where they combine to form bigger globules. The cells of the morula are loose and they become separated from one another under slight pressure. A syncytial layer is formed between the yolk and the convex base of the cell mass. This syncytium is called the periblast.

Stage 10. Blastula (15 hours after fertilization) (Fig. 10)

Changes which ultimately lead to the formation of the blastula now commence. The hemispherical mass of cells which projects from the yolk flattens and, by extending outwards, the periphery of the blastodisk lines up with the periphery of the yolk. The marginal or peripheral cells remain in close contact with the periblast. The central cells of the floor of the blastodisk are raised, forming a central space known as the subgerminal cavity. At the end of segmentation the blastodisk is radially symmetrical but now, due to the fact that the flattening of the cell mass is less expressed in one sector, bilateral symmetry is established. This thicker sector contains the prospective embryonic material and its median plane becomes the median plane of the embryo. The distal part of the thicker sector is the prospective posterior end of the embryo, and the central part of this sector corresponds to the prospective anterior end of the embryo.

Stage 11. Beginning of invagination (22 hours after fertilization) (Fig. 11)

About 7 hours after the appearance of the subgerminal cavity the cells at the edge of the thicker sector begin to invaginate at the limit of the cytoplasm and yolk; this marks the beginning of gastrulation.

Stage 12. Germ-ring (26 hours after fertilization) (Fig. 12)

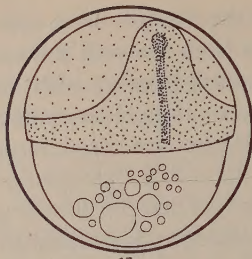
The invagination, beginning at one point, now extends laterally round the edge of the blastoderm and soon spreads to the periphery of the entire disk. The invaginated layer does not extend over the floor of the subgerminal cavity but is confined to the edge of the blastoderm, thus forming a prominent germ-ring. The only part which shows further invagination is the region of the germ-ring which is formed by the thick sector of the blastoderm disk.

Stage 13 Germ-ring reaches the equator of the egg (30 hours after fertilization) (Fig. 13)

As soon as the germ-ring becomes established it moves outward over the yolk towards the equator of the egg, its width remaining constant but its circumference gradually increasing. The blastoderm behind the germ-ring covers an increasing area of the surface of the egg. The part of the embryo which lies anterior to the edge of the blastodermic disk where invagination first began increases in all directions and assumes a triangular shape, the apex pointing towards the animal pole of the egg. A thickening of the embryonic axis soon appears in this region.

Stage 14. Three-quarters of the egg surface covered (36 hours after fertilization) (Fig. 14)

The blastoderm continues to spread over the yolk, and the circumference of the germ-ring decreases as it passes down from the equator of the yolk. The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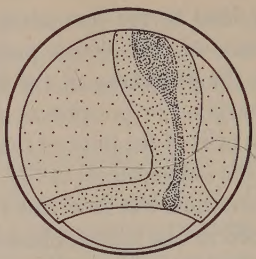
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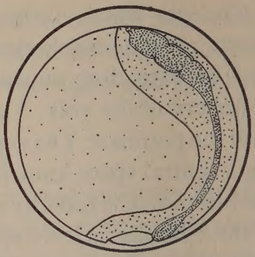
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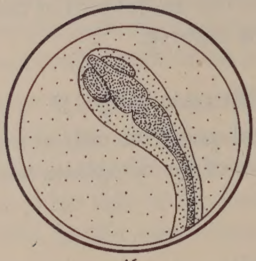
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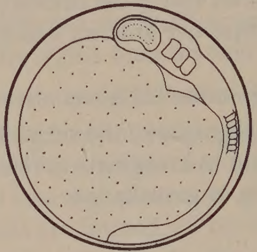
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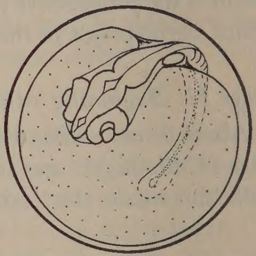
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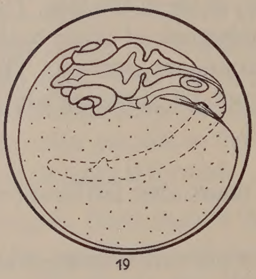
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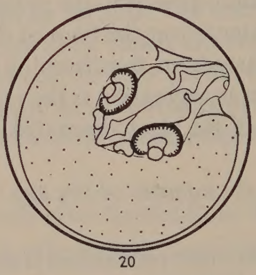
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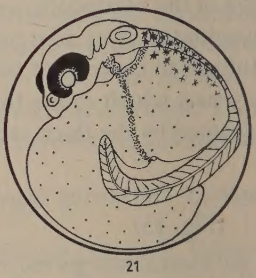
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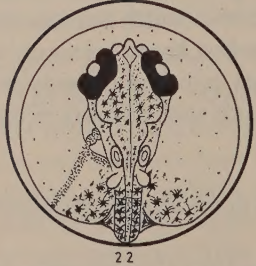
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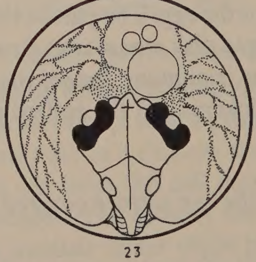
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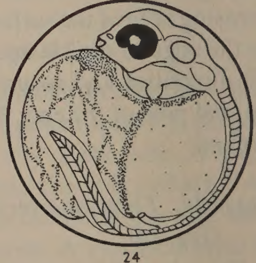
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embryo proper loses its triangular shape and becomes elongated. The neural plate with the brain and nerve-cord areas can now be recognized. At this stage the germ-ring can be compared with the blastopore of anamniote vertebrates.

Stage 15. Small yolk plug (42 hours after fertilization) (Fig. 15)

The overgrowth continues and the blastopore becomes small by the approximation of its lips. This stage can also be recognized by the subdivision of the presumptive brain plate into fore-, mid-, and hind-brain areas. The embryo elongates and narrows transversely.

Stage 16. Closure of blastopore (50 hours after fertilization) (Fig. 16)

In a few hours, after further contraction of the lips, the blastopore closes. The optic lobes appear by lateral outgrowth from the fore-brain. The invaginated parts of the embryo have by now converged towards the embryonic axis. This and the development of the central nervous system cause a thickening of the embryo proper, which now protrudes from the surface of the egg over half its circumference.

Stage 17. First appearance of somites (60 hours after fertilization) (Fig. 17)

When the blastopore closes, the whole germ-ring fuses with the embryo proper which now appears elevated and well demarcated from the yolk. By the development of central cavities the optic lobes become optic vesicles. Soon after this a ventrolateral indentation begins in each vesicle. About six or seven pairs of somites appear in the middle of the embryo. At this stage three blocks of mesodermal tissue are also formed on both sides of the head just behind the optic vesicles.

Stage 18. Lens, brain-vesicles, and otic capsules (70 hours after fertilization) (Fig. 18)

The head of the embryo differentiates further. The optic vesicles are converted into optic cups by the continuation of the ventrolateral indentation and lenses are formed. The brain develops a dorsomedian furrow which widens in the fore- and mid-brain to ventricles which are dorsally open. Lateral to the hind-brain a pair of otic capsules can be recognized. Ventral to the posterior part of the brain the pericardium appears although the heart is not yet visible. The somites increase in number. Kupffer's vesicle is now visible ventrally at the posterior end of the body.

Stage 19. Heart and olfactory lobes (88 hours after fertilization) (Fig. 19)

The brain, particularly the posterior part of the hind-brain, enlarges and a deep constriction appears between mid- and hind-brain. The two ventricles widen further and a third one becomes visible as the lateral folds of the roof of the hind-brain diverge. The otic capsules now possess otoliths. The olfactory lobes develop anterior to the fore-brain. The pericardium enlarges and the beating heart becomes visible on the left side. The distal end of the heart is bent towards the left where it is in open communication with the primitive blood spaces of the yolk sac. Posterior to the blastoporal region the tail develops. Kupffer's vesicle is still present.

Stage 20. Pigmented eye (106 hours after fertilization) (Fig. 20)

The eyes of the embryo become prominent and the choroid fissure narrows. Pigment appears on the peripheral margins of the eyes. The cerebral ventricles in the fore- and hind-brain are still open dorsally but the ventricle in the mid-brain has closed. The olfactory lobes are now cup-shaped. The tail shows occasional muscular movements.

Stage 21. Blood circulation and pigment on body (130 hours after fertilization) (Fig. 21)

The pigment covers a larger area of the growing eye cups which now surround three-quarters of the lens. The ventricle of the hind-brain is no longer noticeable. The heart has a visible auricle, ventricle, and sinus venosus which is distally in communication with the developing yolk sac circulation. The red blood corpuscles can be seen and the heart beats vigorously. The yolk sac circulation begins on the left side of the embryo. Very soon after the appearance of pigment in the eyes, melanophores appear also on the dorsal side of the body and on the neighbouring region of the yolk sac. The gill slits are visible.

Stage 22. Pectoral fins (144 hours after fertilization) (Fig. 22)

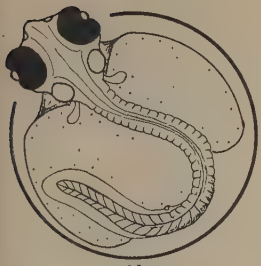
The rudiments of the pectoral fins appear behind the otic capsules. Melanophores spread over the entire eye and body, the yolk sac adjacent to the embryo also being heavily pigmented. The head of the embryo shortens, broadens, and the otic capsules lie more anteriorly. The yolk sac circulation is increasing in area.

Stage 23. Ventricle of fore-brain closed (156 hours after fertilization) (Fig. 23)

The head of the embryo is fully formed and the last cerebral ventricle closes. The pericardium and the heart increase in size; the latter, now bending upon itself, assumes a median position. The yolk sac circulation is completed. The blood from the tail runs into a network of blood capillaries on the entire surface of the yolk; it collects in front of the head in blood islands from where it flows to the heart. The fewer but larger oil globules now lie mainly in front of the head.

Stage 24. Formation of mouth and tail (168 hours after fertilization) (Fig. 24)

At this stage the mouth is formed and, the choroid fissure being closed, the eye cup completely surrounds the lens. The tail is very prominent, possesses a tail fin, and moves vigorously. The pectoral fins vibrate inside the egg capsule. The embryo is almost ready for hatching.



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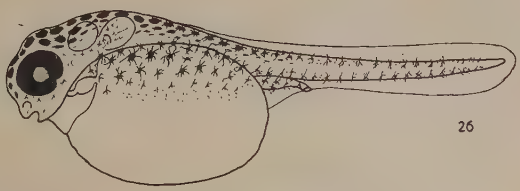

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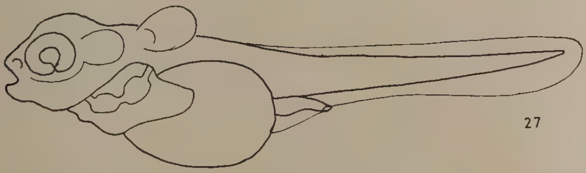

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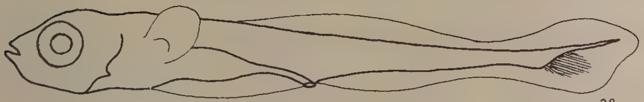

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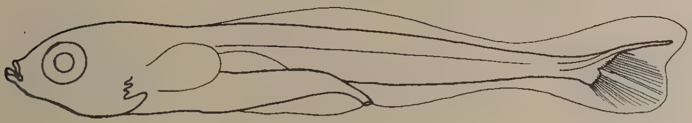

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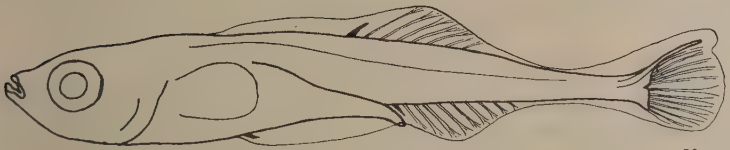

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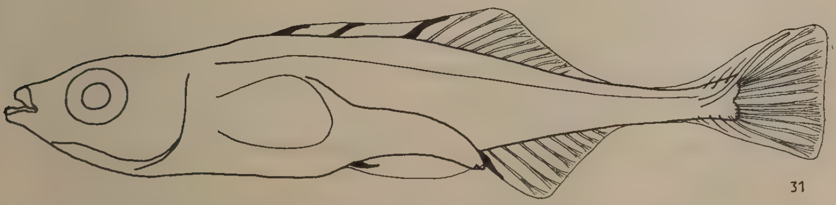

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Stage 25. Hatching (192 hours after fertilization) (Fig. 25)

Now the embryo lashes its tail, the pectoral fins vibrate at high frequency, and the head is pushed against the vitelline membrane or shell of the egg. Later the vitelline membrane ruptures just in front of the head which now protrudes from the egg into the water. A few more movements of the tail free the entire embryo from the egg shell.

Stage 26. Hatched embryo or larva (Fig. 26)

By the time the embryo hatches it is already about 8 days old and measures 3 mm. in length. The larva is transparent and shows a mesencephalic flexure. The mouth is open, the eyes are large, and the pectoral fins constantly move. The hind-gut is visible and opens by the anus just behind the large yolk sac. The caudal axis with its nerve-cord, notochord, and myotomes is straight and possesses a continuous fin fold which extends along the whole dorsal and ventral rim of the tail. There is another small fin beneath the hind-gut between the anus and the yolk. Because of the heavy yolk the larva lies on its side, occasionally swimming swiftly to settle down again at another spot.

Stage 27. Half the yolk absorbed; head straight (1–2 days after hatching) (Fig. 27)

About 24 hours after hatching the larva has a length of nearly 4 mm., has absorbed half its yolk, and swims about freely. The head straightens out which allows the heart and pericardium to unfold.

Stage 28. All yolk absorbed; jaws formed (4 days after hatching) (Fig. 28)

The larva continues to grow by absorption of the yolk which is completely used up 4 days after hatching, giving the larva, now about 6 mm. long, a slender appearance. The fin fold is still continuous, but its caudal part is pointed and develops ventrally mesenchymal rudiments of the caudal fin rays. The jaws develop and the mouth can now be opened and closed. The intestine elongates and the swim bladder appears on its dorsal side. The gill covers also develop.

Stage 29. Heterocercal tail (9 days after hatching) (Fig. 29)

Nine days after hatching the larva reaches about 8 mm. in length and assumes the shape of a fish. The eyes are fully developed and the choroid fissure has disappeared. The fin fold, though continuous, shows signs of division into dorsal, anal, and caudal fins. The caudal end of the notochord grows upwards and the ventral lobe of the caudal fin becomes more prominent, thus forming a heterocercal tail. Its fin rays have appeared. The dorsal and anal fins develop basal mesenchyme thickenings and the pelvic spines are formed. The snout elongates and the lateral line becomes visible.

Stage 30. Dorsal and anal fin rays; tail homocercal (16 days after hatching) (Fig. 30)

Sixteen days after hatching the larva attains a length of about 9·5 mm. The dorsal and anal fins are separated from the caudal fin and are triangular in shape. Dorsal and anal fin rays appear. The third dorsal and the first anal spine are present. The original ventral lobe of the caudal fin increases in size and takes up a symmetrical position, thus forming the homocercal tail. The pectoral fins are strong and show the fin rays, and the pelvic fins are recognizable.

Stage 31. First and second dorsal spines (22 days after hatching) (Fig. 31)

About 22 days after hatching the first and second dorsal spines appear. The young fish reaches a size of about 11 mm. and possesses all typical characters.

ACKNOWLEDGEMENT

I am greatly indebted to Dr. M. Fischberg, under whose supervision this work was done. I should also like to thank Professor Sir Alister Hardy for his help and advice in the preparation of this paper.

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(Manuscript received 29 : xi : 57)

On Regeneration after the Amputation of Abnormal Structures

II. Supernumerary Induced Limbs

by D. R. NEWTH[1]

From the Department of Zoology and Comparative Anatomy, University College London

WITH TWO PLATES

INTRODUCTION

In the course of his work on the determination of urodele limb asymmetry Harrison (1918) found that limbs formed from grafted limb-disks were often too poorly constructed for reliable diagnosis of their symmetry relations to be possible. He hoped that the amputation of such structures might be followed by the regeneration of limbs sufficiently normal in form for his purposes, and in this he was justified in a number of cases. In other cases he was disappointed, regeneration either failed to occur or produced limbs as difficult to diagnose as the originals. As it happens we now know (see Takaya, 1941) that the method has serious limitations in that transplanted limbs can, after amputation, regenerate with a reversal of their original asymmetry. Harrison's results did, however, raise the general problem of the ability of regenerative processes to replace abnormal structures by normal ones. This point was taken up by Swett (1924) who repeated Harrison's experiments upon a larger material. His results were not clear-cut. Normal limbs were usually replaced by normal ones. Limbs which were partly or wholly duplicated were replaced either by normal ones or by hypodactylous hypomorphs, but certainly never by limbs which had the same abnormality as the originals. On the other hand, small, hypodactylous, and deficient limbs were replaced by structures almost equally deficient. This Swett provisionally attributed to nutritional failure since the stumps were so small that he felt that they might well be unable to provide the vascular supply required to support full regeneration.

However, in these experiments the malformed limbs whose regenerative capacity was tested came from normal limb-disks. Consequently, although their primary morphogenesis was, by definition, abnormal, their initial determination to form a limb was not. Their abnormalities were thus probably due to contingent

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factors consequent upon grafting, and not necessarily still operative during later regeneration. A quite different situation can, in principle, be considered to be involved in an isolated experience reported by Filatov (1927). He had induced supernumerary limbs in the urodele flank by the implantation of otic vesicles. One of his induced limbs had three digits only, the middle one having a forked terminal phalanx. This limb was bitten off through the carpus by another larva and the regeneration that ensued produced a replica of the abnormal hand. In this case it could be argued that the abnormality of the supernumerary limb reflected an aberration in the process of induction and that the morphogenesis of the regenerate was controlled by influences which originated during induction. Balinksy (1933) was later to report three further cases of regeneration in induced limbs of abnormal structure. In two of them no improvement was effected by regeneration, in the third some progress towards normal structure was obtained. More recently Breedis (1952) has produced abnormal accessory limbs, sprouting from the upper arm, by the injection of carcinogens into the limbs of adult *Triturus*. Subsequent amputation of the whole limb proximal to the secondary growth produced, in each of 6 cases, a normal regenerate. In 15 other cases the accessory limb alone was removed, to be replaced by regenerates which 'varied, but in general . . . either approximated the structure of the amputated growth or were less complex'.

Now we know from the fundamental findings of Weiss (1925, 1926) that a normal regenerate can be formed from a urodele limb whose cross-section at the plane of amputation is highly abnormal (see also Goss, 1956 *a*, *b*, 1957). Further, Polejaiev (1936) has shown that stump tissues whose structural integrity has been destroyed by mincing can support the formation of normal regenerates. But in these cases the abnormality of the stump is a product of experimental interference long after the primary morphogenesis of the limb has been normally accomplished, indeed, at a time immediately before amputation. They do not, therefore, rule out the possibility that limbs whose earliest determination has been abnormal should replicate their abnormalities in regeneration. It is true that to do so they would presumably have to override the influence of the 'limb-field' whose extraordinary potency in post-embryonic amphibians is evidenced by the results of Farinelli-Ferruzza (1957) and Holtfreter (1955). These authors find that tails induced in or transplanted to the flank may be transformed into limbs with or without the stimulus to secondary morphogenetic processes provided by amputation.

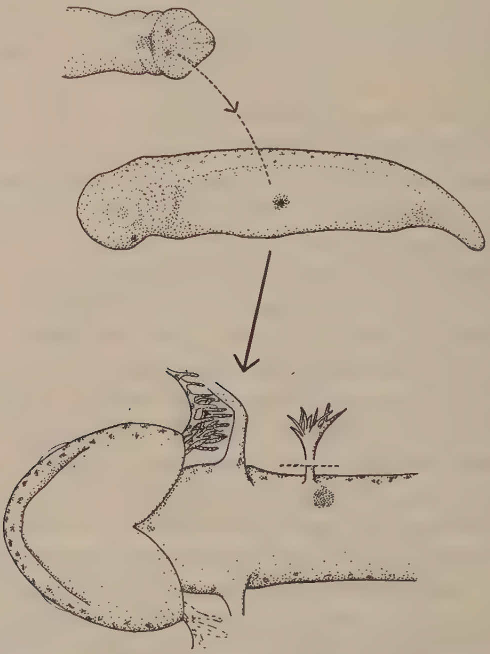
It must, however, be added that Rostand (1955) has obtained normal regenerates from hind-limbs of *Rana* tadpoles that were destined to become highly abnormal, though as yet the cause of the abnormality with which he was dealing (discovered in a wild population) remains enigmatic.

The present paper reports experiments designed to extend the available material on the regeneration of abnormal induced supernumerary limbs in urodeles. From them it was hoped to find out whether such limbs behaved as had

Filatov's single case or were, by contrast, replaced by normally formed regenerates.

MATERIALS AND METHODS

Embryos of *Triturus helveticus* were used in these experiments. Following Glick (1931) nasal placode was used as the inductor, the operation (see Text-fig.) being performed with donors at stage 25 (Glaesner, 1925) and the hosts at stage 27. Procedures were standard. The hosts were reared communally until they started feeding and thereafter individually. To avoid the difficulties and dangers of rearing animals through metamorphosis all experimental animals were killed at its onset. The material derived from animals in which induction occurred was preserved for histological examination.



Schematic representation of the experiment performed. A newt embryo receiving a homoplastic graft of nasal placode in the flank reacts by the formation of a supernumerary limb which is subsequently amputated.

RESULTS

Of 163 hosts the great majority showed no signs of forming induced structures, the percentage of success achieved being, in fact, much less than that obtained by other workers on other species (see Takaya, 1941, for details). Some of the failures were later sectioned, but nothing in the histology of the graft region that might account for their lack of response was found. In 19 animals only did the flank of the host react by producing an outgrowth. The time of first appearance of the induced structures was rather late when compared with Balinsky's (1937) animals. Of these 19 animals 4 produced hyperdactylous limbs, a further 4 produced normally formed ones (and one of these animals showed a double induction, both its supernumerary limbs being normal in form), and 11 animals had inductions which were hypomorphic to a greater or lesser degree. These varied from limbs with one, two, or three well-formed digits to blobs and protrusions which had none.

Twenty amputations were thus performed. In each case the animal was carefully observed for a week before amputation to ensure that morphogenesis in the induced limb was not proceeding still. The plane of amputation, in cases where a recognizable limb was concerned, was as near to the flank as was consistent with confidence that regeneration would occur at all. In those inductions which were mere bumps no such discretion could be exercised, and in the smallest of them the operation could only by courtesy be called amputation. Wherever possible the amputate was preserved, but in three cases involving small protuberances the tissues removed were lost.

In 4 animals (F8, F9, D8, D10) no regeneration occurred. The other 13 all provided regenerates of some sort. Where the regenerate formed was a limb of normal appearance (i.e. with the four digits characteristic of a forelimb) the animal was left without further interference until metamorphosis, when it was killed and preserved. Where the regenerate was not a normal limb it was itself re-amputated after it had completed its morphogenesis. The second regenerates thus formed were approaching the completion of their morphogenesis when the onset of metamorphosis made the sacrifice of their owners necessary.

The major results of these operations are shown in the Table, and some of them are illustrated in the Plates. Briefly, they suggest that normal or slightly abnormal induced limbs will generally be replaced after amputation by normal ones, but that if the original abnormality is very great the regenerate may be intermediate between the original induction and the normal. In such a case progress towards normality may be carried further by a second regenerative process. The significance of these generalizations, and the exceptions to them, will be discussed below, but first it is necessary to comment further on the results themselves.

In the first place it might seem that the number of digits possessed by an induction or regenerate is not necessarily an adequate measure of its departure from or approach to normality. In practice, however, it is a reliable index except in cases of extreme hypodactyly. This is so because all those inductions which had three or more digits possessed a single humerus, radius, and ulna. Only the autopodium is markedly abnormal and this abnormality shows itself in the presence of too many or too few elements and can thus be quantitatively described. But it must be confessed that in the smaller inductions (e.g. that of F4, see Plate 2) the skeletal elements of the stylopodium and zeugopodium are often represented by a single cartilage of no obvious morphological status. In such cases the degree of hypodactyly may be an indication of the extent of abnormality, but does not fully describe it.

It would be of great value to be able to assess the morphological significance of the hyperdactylous limbs in terms of the level of reduplication. Do their digits, for example, fall into groups of four, some groups perhaps incomplete, but each representing a distinct hand? Only in one case, the first regenerate of animal F2 (see Plate 1, fig. B) is it possible to say with confidence that the 8 digits there displayed do represent two hands. In all other cases it proved impossible to reduce the sometimes chaotic arrangement of elements in the carpus and metacarpus into morphological order. This does not, of course, allow us to deny that they represent separate autopodia whose integrity has become obscured.

Finally, it is important to stress that the plane of amputation was in all cases chosen to be as proximal as practicable in order that such duplications or defects as might appear in the distal part of the limb should not be represented there. This aim was realized and the plane of amputation always passed through the humerus, or through a single cartilage lying at the base of the induction. This does not mean, however, that the cut surface was anatomically normal. Induced limbs, even when they appear outwardly normal in form, tend to have a very poorly developed musculature. This fact may well reflect quantitative inadequacy of their innervation, and it has the consequence that the blastema forms

TABLE

*The appearance of induced limbs and of their regenerates**

Animal	*Induction*	*First regenerate*	*Second regenerate*	*Comment*
F2	12	8	4	See Plate 1, figs. A–C
F1	10	5	4	See Plate 1, figs. D–H
D1	9	5	4	
D3	7	4	—	
F5	4	4	—	
D7	4	4	—	
D5	4	4	—	
F3	4	4	—	Two limbs induced by one graft. See Plate 2, figs. J–L
	4	1	—	
D9	3	4	—	
F4	2	4	—	See Plate 2, figs. M, N
D5	0	1	4	See Plate 2, figs. O, P
F7	1	1	0	
D2	1	0	1	
D6	3	1	0	
F6	1	0	0	
F8, F9 D8, D10	0	×	—	

* As judged by the number of digits. 0 = a digitless protuberance; × = failure to regenerate; — = no second amputation performed.

on a stump in which the proportion of cartilage to muscle is abnormally high. This can be seen in Plate 1, fig. G. The regenerates generally show an increased representation of muscle tissues though, as Plate 1, fig. H shows, the improvement is not necessarily great. Nevertheless, it is noteworthy, and possibly significant, that the approach to a more normal outward form in originally hyperdactylous limbs goes *pari passu* with an approach to a more normal balance between skeleton and muscle at the plane of amputation.

DISCUSSION

It is not surprising that regeneration failed to occur after the amputation of many of the smaller protuberances. On the one hand, they may not have been the products of morphogenetic activity, but only bumps representing the mechanical accommodation of the hosts to the grafts themselves. On the other hand, they were, of necessity, amputated so close to the flank that even had they been normal limbs regeneration might well have failed. We may also dismiss from further consideration those cases in which regenerates were formed but were less nearly normal than the original structures. In all but one of them it is possible that the stump tissues were quantitatively inadequate for the support of full regeneration. In one apparent exception (animal F3, see Plate 2) one of a pair of induced limbs failed to regenerate. Here we must take into account the possibility that competition between the two regenerating systems permitted only one to complete its morphogenesis.

The remaining cases, though admittedly few in number, would seem worthy of comment even if they formed a small part of a much larger material, the bulk of which had behaved differently. They all provide evidence for the existence of factors operating during regeneration and tending to the production of a species-specific formation—a normally constructed limb. We may accept that two such factors are involved. The first consists of that component of the genetic information of the fertilized egg that has been transmitted without change in the cell-lineage of the blastema cells. The second is that property of the amphibian flank which guides morphogenetic processes of many sorts to the formation of limbs. When we consider the induction of supernumerary limbs in embryos (Balinsky) or in larval and adult stages (Kiortsis); the regeneration of normal limbs from abnormal stumps of limbs (Weiss) or induced tails (Holtfreter); and the transformation of transplanted tails into limbs (Farinelli-Ferruzza) there is ample excuse for expecting abnormal supernumerary induced limbs to be replaced by normal ones.

It is more difficult to conceive of factors tending to the reproduction by regeneration of individual abnormalities. Nevertheless, the failure of several of the cases reported in this paper to achieve normality after their first regeneration suggests that genetic and limb-field influences are having to work on material which has a tendency to replicate the original structure however malformed. The efficacy of this tendency appears to be greater the more abnormal the amputated structure, as evidenced both by the degree of abnormality of the first regenerate and the number of regeneration generations required to restore normality. Of the four animals involved one (D5) started with a small, digitless, induction which after two amputations had just achieved the dignity of a limb (see Plate 2). Its behaviour could, quite reasonably, be explained in terms of simple quantitative inadequacy of the original stump. The first blastema, although too small to provide a proper limb, may have given rise to a structure which was large enough to do so after a second amputation. The significance of this case, taken together with animals F4 and D9, is mainly that they show that there is no general tendency for the regenerates of induced limbs to be hypomorphic. The explanation of the three cases (F1, D2, and D1) in which hyperdactylous limbs were replaced by more nearly normal ones cannot thus be due to any such cause.

Indeed, if these cases stood quite alone one might be tempted to ignore them as of little consequence. They should, however, be considered in conjunction with the results of Breedis and Filatov referred to above, and possibly also with some of the results of Takaya. Filatov's single case cannot be given very much weight, if only because the experiment was performed by a urodele larva which presumably ate the amputate. It cannot be excluded that the cut surface of the stump, at the level of the carpus, reflected the three-digited condition of the induced hand. Only the forked terminal phalanx remains to be explained. Takaya's regeneration experiments included a number in which duplicated limbs, formed after transplantation of limb-buds, were amputated proximal to their level of bifurcation. Usually they were replaced by single structures, but in a few cases the duplication was replicated in the regenerate. Two points must be made in considering these cases. Firstly, it is not clear from Takaya's description that the level of amputation lay proximal to any duplication of internal structures. As we know from Goss's (1956*b*) work, such internal duplication may result in partial duplication in the regenerate. Secondly, Takaya's material is not necessarily comparable to that presented in this paper since he was working with transplanted normal limb-buds. The cases reported by Breedis, on the other hand, are more directly comparable and gave results which are wholly consistent with the behaviour of my material. It is only to be regretted that he did not perform second amputations on the regenerates formed by his supernumerary limbs.

Taking the results of Breedis and those reported here together, we may give provisional acceptance to the view that there are factors working against normogenesis in the regeneration of highly polydactylous supernumerary limbs. Further work is clearly necessary before their existence can be regarded as established, and in particular it would be important to know whether the process by which normalizing tendencies come to dominate them is dependent upon the passage of time alone or whether the exercise provided by successive regenerations is critical. Would, for example, the supernumerary limb of animal F2 have been replaced by a normal one after only a single regeneration if the amputation provoking it had been delayed to the time at which the second amputation was, in fact, performed?

But if such factors do exist it is important to stress that they are not necessarily mysterious or intangible. They might, it is true, be a consequence of the blastema cells' experience of having undergone an abnormal primary morphogenesis. Such experience has been invoked to explain the consequences of amputating abnormal amphibian tails, but with little justification (see Newth, 1958). It is more likely that we are dealing with behaviour that is to be explained in terms of stump structure. Thus it was pointed out above that hyperdactylous induced limbs are characterized by the possession of a poorly developed musculature and a well-developed skeleton. Now Nassonov (1936) has produced evidence for the capacity of cartilage and cartilage extracts to stimulate morphogenetic activity in post-embryonic amphibians, and Goss (1956 *a*, *b*) has recently shown that additional skeletal elements in the stump may cause the formation of additional elements in the regenerate. It is not wholly impossible that a drastic raising of the proportion of skeletal to muscular tissue in the stump might lead to hyperdactyly in the regenerate. This, and the effects of an innervation which is both qualitatively and probably quantitatively abnormal in induced limbs, would have to be investigated before recourse was had to more exotic explanations.

SUMMARY

1. Supernumerary limbs induced in the flank of larvae of *Triturus helveticus* by homoplastic grafts of nasal placode are frequently abnormal in structure. Some are highly polydactylous, others hypodactylous, and yet others are mere protuberances whose character as limbs cannot be assumed from their appearance *in vivo*.

2. The amputation of such induced formations at a level close to the flank of the larva is usually followed by regeneration. The regenerates so formed are, in most cases, more nearly normal in structure than the original inductions they are replacing. A second amputation and regeneration may completely restore normality.

3. The possibility of explaining these results in terms of the known peculiarities of supernumerary induced limbs is discussed.

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EXPLANATION OF PLATES

PLATE 1

Figs. A–C. The induction (1st amputate), first regenerate (2nd amputate) and second regenerate respectively of animal F2.

Figs. D–F. The induction, first regenerate and second regenerate respectively of animal F1. In fig. F the normal forelimb is also shown.

Figs. G, H. Transverse sections through the first and second amputates respectively of animal F1 taken very close to the plane of amputation.

(Magnifications are the same for figs. A, B, D, and E, for figs. C and F, and for figs. G and H.)

PLATE 2

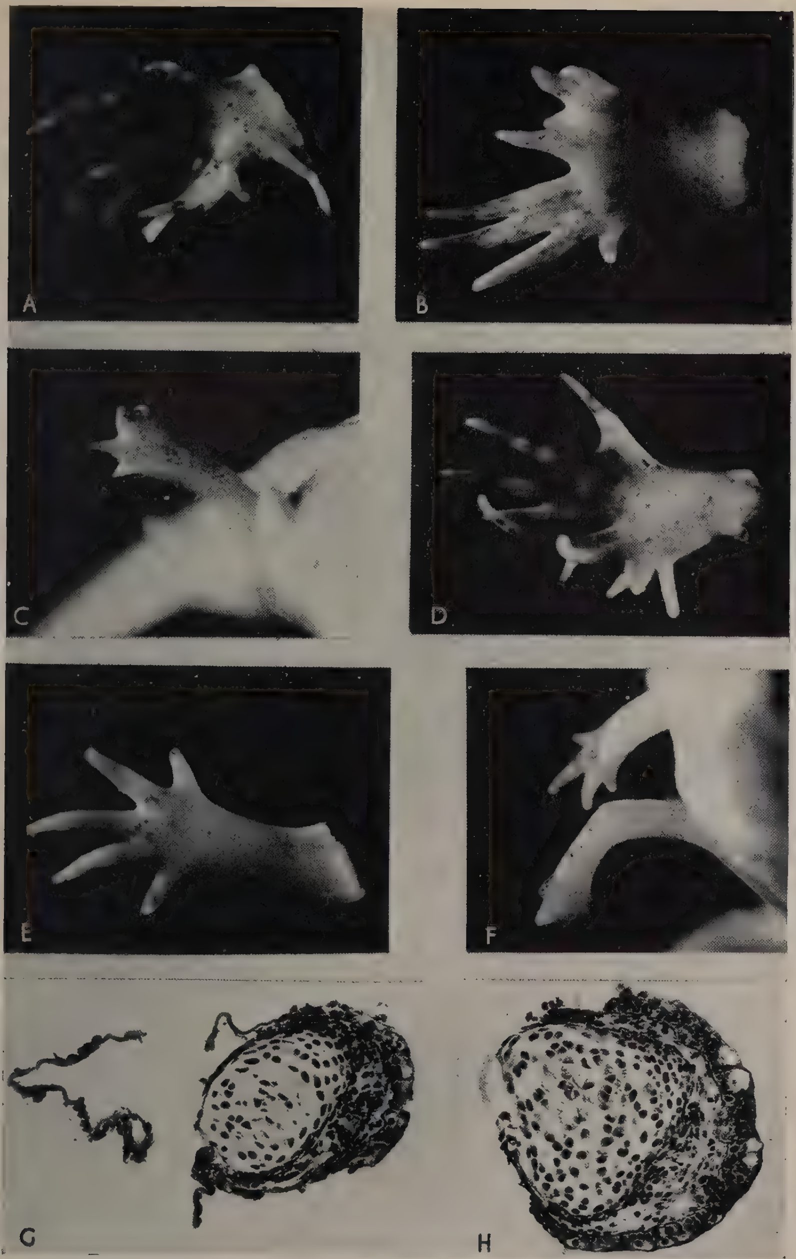
Figs. J–L. Two independent inductions associated with a single graft were formed in animal F3. On amputation the dorsal one (fig. J) was replaced by a spike (fig. L) while the ventral one (fig. K) was replaced by a well-formed limb.

Figs. M, N. The amputated induction (fig. M) and the regenerate formed (fig. N) of animal F4.

Figs. O, P. The second amputate and the second regenerate respectively of animal D4. The first amputate, a digitless 'bump', was too small to be preserved. The normal forelimb in fig. P has been cut away to display the regenerate.

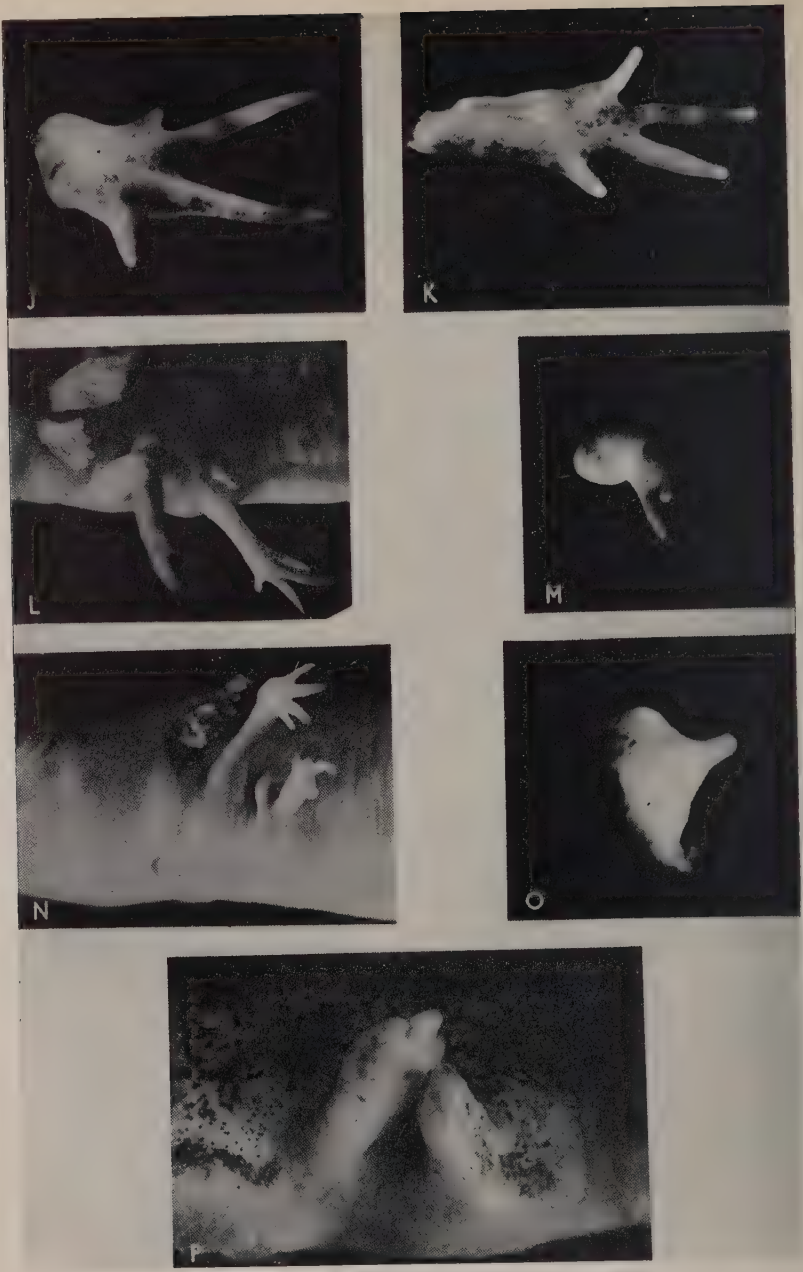
(Magnifications are arbitrary.)

(Manuscript received 6 : xii : 58)



D. R. NEWTH

Plate 1



D. R. NEWTH

Plate 2

Experimental Tetraploidy in Newts[1]

by M. FISCHBERG[2]
From the Department of Zoology and Comparative Anatomy, Oxford

WITH ONE PLATE

INTRODUCTION

It is very easy to change the number of whole chromosome-sets in Amphibia. Amphibia are better suited for this kind of work than other vertebrates. The technique of changing the number of chromosome-sets, combined with hybridization, which is also easily achieved, can contribute to a wide range of problems. In the course of certain investigations it is of importance to know the origin of the chromosome-sets present. In this case newts should be used in preference to anurans, because the contribution of chromosome-sets seems to be more regular in Urodela than in some species of Anura, where in experiments on artificial parthenogenesis larvae with from one to six chromosome-sets were produced (Kawamura, 1939). In this paper only the Urodela will be discussed.

Methods are known for producing haploid gynogenetic and androgenetic newt embryos or larvae. It is also possible to produce haploid androgenetic hybrids which possess only the cytoplasm from one species and one chromosome-set from another. Unfortunately, all these haploid types are not viable. Recently a method has been developed to produce diploid gynogenetic newts (Fischberg, 1947). These animals develop from eggs containing a diploid set of maternal chromosomes and are able to survive much longer than the haploids.

In 1939 Fankhauser & Griffiths discovered a simple method which enabled them to produce triploid newts in great numbers. Although some biologists have been working on polyploidy in amphibians since 1940, no efficient method had been found which made it possible to obtain tetraploid newts in considerable numbers. In order to study the effects of tetraploidy one had to rely on the very few tetraploid larvae which occurred in experiments designed to produce triploidy. But this number only amounted to about 0·7 per cent. of larvae which developed from temperature-treated eggs, or only about 0·15 per cent. of eggs treated in earlier experiments (Fischberg, 1948). The rate of spontaneous occurrence of tetraploidy is still lower.

The highest percentage of tetraploidy that had been obtained so far was from

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[1] This paper has been written as a tribute to Professor F. Baltzer.

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the cross of triploid Mexican axolotl females with diploid males. Fankhauser & Humphrey (1950) observed that from 2,027 fertile eggs, 48 tetraploids or near tetraploids developed. This amounts to 2·4 per cent. of all fertile eggs and to 10·79 per cent. of all individuals of which the chromosome number was determined. This second percentage is, according to Fankhauser & Humphrey, artificially high, as these authors think that they were able to recognize all tetraploids but not all hyperdiploids ($2N+1$ to $3N-1$), the frequency of which they judge as being close to 95 per cent. of all surviving larvae.

This method of producing tetraploid larvae is rather laborious and gives one no freedom to choose the urodele species, as the only urodele species so far found fertile in the triploid state is the Mexican axolotl. Kawamura (1951) showed for a second species (*T. pyrrhogaster*) that offspring from triploid individuals crossed with diploids can be obtained in small numbers by means of artificial insemination. But no tetraploid larvae were found amongst them.

The first attempts to produce tetraploidy using a technique similar to that by which triploidy is produced, i.e. by temperature treatment of newt eggs at the appropriate stage, were carried out by Böök (1943), by Fankhauser & Watson (Fankhauser, 1945), and by Sanada (1951). Böök subjected the fertilized eggs, after the 2nd maturation division but before the first cleavage, to low temperatures. He thinks that this treatment inhibits the anaphase movements of the chromosomes of the first cleavage division and thus creates a tetraploid nucleus in the uncleaved egg. All embryos were complex mosaics or, as Böök calls them, 'multiform aneuploids'. They arose, in the opinion of this author, as a consequence of a multipolar mitosis caused by the presence of 4 centrosomes.

Fankhauser treated his eggs shortly before the first cleavage division with a temperature of 36° C. He was able to suppress the first cleavage of the egg, but at the time of the second cleavage these eggs divided simultaneously into 4 cells and gave rise to normal diploid larvae. Watson treated the eggs with colchicine solutions but was not successful either.

Sanada was the only one who actually found a method which produces tetraploid Amphibia without having to rely on fertile triploid newts. He exposed the eggs of *T. pyrrhogaster* from 6 hours to 7 hours 50 minutes after artificial insemination for 15–31 hours to a temperature of 0° to -1° C. He states that 'the mortality of the treated eggs was considerably high' and that only a 'small number of them developed into larvae' (Sanada, 1951, p. 35). Ten of these (or 6·3 per cent.) were tetraploids. It is quite clear that the practical value of this method cannot be determined unless one knows the mortality. The 10 tetraploids might correspond to anything between 3 per cent. and 0·5 per cent. of the eggs treated.

PRELIMINARY EXPERIMENTS

Encouraged by the fact that it was possible to produce tetraploid pre-implantation mouse embryos by heat treatment (Fischberg & Beatty, 1950; Beatty &

Fischberg, 1952), I began some preliminary experiments on newts in 1950. Eggs of *T. palmatus* (*helveticus*) and some eggs of *T. vulgaris* were exposed to temperatures ranging from 35° to 38° C., for a duration of 10–12 minutes. Every quarter of an hour from 3 to 6¾ hours after insemination a different series of eggs were treated in this way.

All eggs were artificially inseminated. The eggs of one female were therefore of exactly the same age and were always kept at the same temperature and in the same Petri dish. Nevertheless, even before the treatment the eggs of each female did not develop at exactly the same rate. At the time when the first cleavage division should occur I always found some 2-cell stages, some eggs which showed the first signs of cleavage and some entirely uncleaved (but definitely fertilized) eggs in the same Petri dish.

1,060 eggs were treated before the first sign of cleavage appeared in any of these series of eggs. Only 4 embryos reached advanced developmental stages, but their chromosome number could not be counted. From 1,019 other eggs, of which at least one in each Petri dish was a 2-cell stage or showed the first cutting in of the cleavage furrow, 25 advanced embryos or larvae were obtained. The chromosome number could be determined in the epidermis of the amputated tail tips in 15 of these: 4 were diploid, 5 were tetraploid, 4 were diplo-tetraploid mosaics, 2 were multiform aneuploid.

In spite of the very high mortality, this result indicated that tetraploidy can be produced in newts by application of a 'hot-shock'. All the 5 tetraploid embryos or larvae developed from eggs which were treated at a temperature of 36°–37° C.

FINAL EXPERIMENTS

In order to improve the technique we had now to find out which of the three treated stages of the eggs present in each of the Petri dishes gave rise to the tetraploid larvae. We divided the eggs of each female into three groups which were of the same age but at different developmental stages: pre-cleavage stages, beginning of 1st cleavage stages, 2-cell stages.

All the eggs of one female were treated simultaneously at the same temperature. The mortality was still high in all three groups, but we see from Table 1 that the mortality was highest in the pre-cleavage stages, a little lower in the eggs which had begun to cleave and still lower in the 2-cell stages.

Table 2 demonstrates the result of the chromosome counts in the epidermis of the tail tips. The 82 analysed embryos or larvae, characterized by their chromosome number, belong to five classes: normal diploids, triploids, tetraploids, diplo-tetraploid, and triplo-tetraploid mosaics. The triploid and the mosaic larvae are so rare that we can neglect them for the time being and only consider the rows for diploid and tetraploid individuals. On the left of the number of larvae we find the percentage of all treated eggs which developed into diploids or tetraploids. On the right of the same number the percentage of analysable newts which were diploid or tetraploid is indicated.

The high mortality of the treated pre-cleavage stages, and also the rather low percentage of analysable newts which were tetraploid, exclude the treatment of pre-cleavage stages as a working method for the production of tetraploidy. The same holds for the eggs which were treated when beginning cleavage.

TABLE 1

Mortality of eggs and embryos of T. palmatus *and* T. vulgaris *after treatment with heat*

(Stages Gl. 32 and 41 refer to Glaesner, 1925)

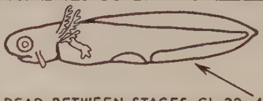
	TRITURUS PALMATUS						TRITURUS VULGARIS			
TREATED STAGE	PRE-CLEAVAGE STAGE		BEGINNING CLEAVAGE		2-CELL STAGE		2-CELL STAGE		TOTAL	
	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
TOTAL EGGS TREATED	681	100	132	100	262	100	62	100	1137	100
DEAD BEFORE STAGE GL. 32	671	98·53	114	86·36	214	81·68	42	67·74	1041	93·32
 DEAD BETWEEN STAGES GL. 32–41.	3	0·44	3	2·27	11	4·20	9	14·52	26	2·29
HATCHED LARVAE	7	1·03	15	11·36	37	14·12	11	17·74	70	6·16

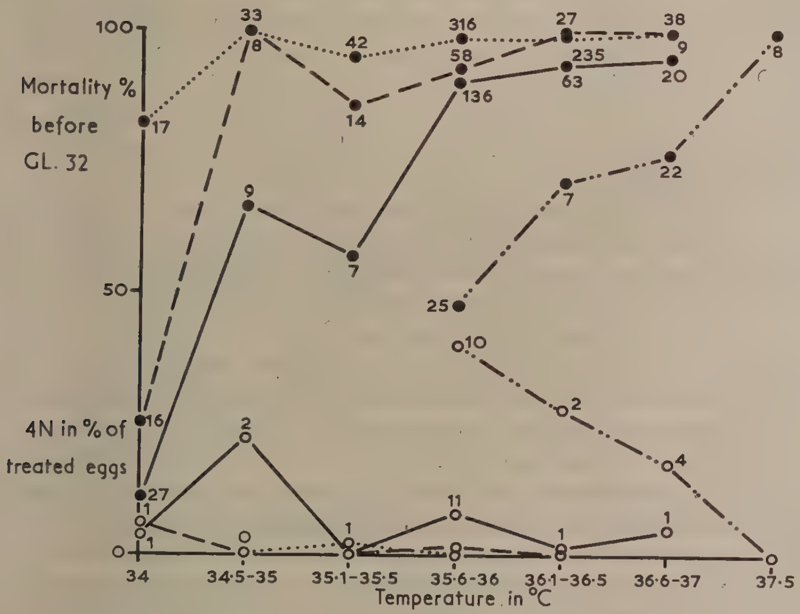
TABLE 2

Effect of heat-shock treatment of the eggs of T. palmatus *and* T. vulgaris *on the chromosome number of the resulting embryos*

	T. palmatus									*T. vulgaris*			
Treated stage	*Pre-cleavage stage*			*Beginning cleavage*			*2-cell stage*			*2-cell stage*			*Total*
	(%)		(%)	(%)		(%)	(%)		(%)	(%)		(%)	
Total eggs treated	100	681	..	100	132	..	100	262	..	100	62	..	1,137
Analysable newts	0·9	6	100	11·4	15	100	17·2	45	100	25·8	16	100	82
Diploid . .	0·6	4	66·7	9·1	12	80·0	10·7	28	62·2	..	..	..	44
Triploid . .	0·2	1	16·7	..	..	..	..	..	..	..	..	..	1
Tetraploid . .	0·2	1	16·7	1·5	2	13·3	6·1	16	35·6	24·2	15	93·7	34
Diplotetraploid mosaic eggs .	..	..	..	..	..	..	0·4	1	2·2	1·6	1	6·3	2
Triplotetraploid mosaic eggs .	..	..	..	0·8	1	6·7	..	..	..	..	..	..	1

But the results of the experiments in which eggs were treated at the 2-cell stage are satisfactory. Seventeen per cent. of the treated eggs survive long enough to be analysed by the tail-tip method and 35·5 per cent. of these are tetraploid. This corresponds to 6 per cent. of all the treated eggs. In the experiments with *T. vulgaris*, which were carried out in 1952 by the late Miss E. Pendleton, the effect of the treatment was even more successful. Twenty-four per cent. of all treated eggs, or 93·7 per cent. of the analysable individuals, were tetraploid.

The method would be ideal if all surviving larvae were tetraploid and no diploids occurred. Then one would not lose time in counting the chromosomes of diploids. It is therefore necessary to make a compromise between relatively low mortality of the eggs and a high proportion of tetraploids compared with diploids.



TEXT-FIG. Effect of temperature of heat-shock on mortality (4 upper curves) and on the production of tetraploidy (4 lower curves). The numbers on the 4 upper curves represent the total number of eggs treated at the particular temperature, while numbers on lower curves represent the actual number of tetraploid embryos and larvae resulting from the treatment. Gl. 32 is a developmental stage according to Glaesner (1925). · · · · · · = *T. palmatus* eggs treated before cleavage began; - - - - - - = *T. palmatus* eggs treated at beginning of cleavage; ——— = *T. palmatus* eggs treated at 2-cell stage; – · · – · · – = *T. vulgaris* eggs treated at 2-cell stage.

The Text-fig. shows the effect of temperature on mortality and on the frequency of the occurrence of tetraploidy. Many numbers in these curves are too small to be of significance, but it is possible to get a general picture of the temperature effect as 1,137 eggs are included in this figure.

If we look first at the four top curves, we see that the mortality rises with increasing temperature. This holds for the eggs of *T. palmatus* which have been treated at the pre-cleavage stage, as well as for those treated during the beginning of cleavage or during the 2-cell stage, and also for those of *T. vulgaris*, treated at the 2-cell stage. The mortality is highest after treatment of pre-cleavage stages and lowest after treatment of 2-cell stages. *T. vulgaris* shows a considerably lower mortality than *T. palmatus*. It is further evident that treatment with a temperature of 35·6°–36·0° C. is the most effective, the mortality not being too high and the number of tetraploid newts being almost equal to the number of survivors.

THE MECHANISM

Previously, it was generally believed that tetraploidy in Amphibia could best be produced by inhibition of the anaphase movements of the chromosomes due to a treatment during the metaphase of the first cleavage division, and hand in hand with this, or as a consequence of it, the cytoplasmic cleavage would be inhibited. Tetraploidy would then result by doubling the chromosome number in the uncleaved egg.

The facts, however, are quite different. As we have seen, the eggs are best treated immediately after completion of the first cytoplasmic cleavage. Cytological examination showed that at this stage of cytoplasmic cleavage each of the two cells contains an interphase nucleus (Plate, figs. A, B).

During heat treatment, or very soon afterwards, the cytoplasmic cleavage is reversed in most eggs. An uncleaved egg with two interphase nuclei results. About 3 hours after the heat treatment the egg divides again into two cells. In most cases the new furrow does not correspond with the position of the primary, vanished furrow. Within these 3 hours the egg becomes tetraploid. We do not yet know if the two interphase nuclei fuse, or if two mitoses fuse at metaphase, or if the chromosomes of the two mitoses migrate during the second anaphase to only two poles instead of four. The presence of diplo-tetraploid mosaic newts (6 in all, 4 from the preliminary experiments and 2 shown in Table 2) suggests a fourth possibility. These mosaic larvae seem to originate from eggs in which only one of the two nuclei, present at the time of treatment, became tetraploid while the other remained diploid. Development would then proceed with both the diploid and the tetraploid nuclei. This seems to indicate that tetraploidy results from the independent doubling of chromosomes of both nuclei during the second mitotic division. This could be brought about by the failure of anaphase movements resulting from inhibition of centrosome doubling or inhibition of centrosome movements. The mechanism which leads to tetraploidy is under cytological investigation.

THE TETRAPLOID NEWTS

Tetraploid urodeles have previously been described in some detail (Fankhauser, 1945; Fischberg, 1948; Sanada, 1951). I will therefore give only a sketch of their main characteristics. Diploid individuals of *T. palmatus* and *T. vulgaris* both have 24 chromosomes in each cell and tetraploid individuals have 48 chromosomes (Plate, figs. C, D). The volume of the nuclei and cells of tetraploids is about twice the volume of diploids. However, organ and body size remain more or less normal. This is achieved by a reduction of the cell number in polyploids, proportional to the increase in cell size (Plate, figs. E, F). Each organ therefore contains only about half the number of cells as compared with diploids.

The viability of tetraploids is generally lower than that of diploid or triploid newts. Many of them die as old embryos or larvae, but many may survive for some years, as shown in the case of the Mexican axolotl (Humphrey & Fankhauser, 1949; Fankhauser & Humphrey, 1950) and for *T. pyrrhogaster* (Sanada, 1951). Apart from an occasional axial curvature and reduced growth rate in many of the older animals the tetraploids are of normal appearance.

DISCUSSION AND OUTLOOK

We have seen that the technique for producing tetraploid *T. palmatus* and *T. vulgaris* is an extremely simple one. We can neglect the time after fertilization and the temperature at which the eggs are kept. We have only to pick out the early 2-cell stages, which are recognizable with the naked eye, and to transfer them to water at 35·6°–36·0° C. for 10 minutes, and tetraploid newts will be produced.

I am certain that it will now be possible to produce tetraploidy in most other urodele species. We have, however, to consider two factors which might be of importance if experiments of this kind are carried out with other species. Firstly, the result of the experiment depends on treatment at a specific temperature. The Text-fig. demonstrates that the eggs of the two species used in the experiments show a different mortality when exposed to the same temperature. *T. vulgaris* has the lower mortality, which might perhaps be expected from the geographical distribution of this species, which is as a whole more exposed to extremes of temperature than *T. palmatus*. The difference in mortality and the fact that different species live in different climates indicates that for the production of tetraploidy the effective temperature is likely to vary from species to species.

Secondly, the stage at which the eggs have to be treated is also very specific. In our species the first cleavage furrow has to close, or almost to close, at the vegetative pole of the egg. Cytological observation has proved that this externally visible stage of the egg corresponds with the presence of two nuclei, which are in middle or late interphase. I believe that it is important that the later interphase stages of the nuclei are treated in order to obtain tetraploidy. It can be assumed that not all species of Amphibia produce eggs in which this specific stage of the nuclei corresponds with the described stage of externally visible cleavage. The size of the eggs and other factors might be of importance.

The reason why I described the method of producing tetraploidy in newts in considerable detail is not because the technique, as such, is of special interest, but because I think this method might be of great help in the investigation of a wide range of problems. It is now possible to produce tetraploid newts in great enough numbers to carry out physiological research. Cell size and relative surface of cells are believed to be of importance for physiological processes. With the possibility of producing polyploid individuals in Amphibia, such theories can be tested experimentally on ideal material and the physiology of polyploid animals can be investigated (Douglas, in manuscript). Tetraploid organisms can be of the same species and age as diploids, they can have the same body and organ size as the diploid controls, but they are composed of much fewer, larger cells.

It is now possible to produce tetraploid hybrids in Amphibia (Fischberg, unpublished) which are of genetic interest and might also help to throw some light on the part hybridization plays in vertebrate evolution.

It can be assumed that if a method enables us to make tetraploid animals out of diploids the same method will allow us to make diploids out of haploids. This would be of great interest because all haploids so far produced were non-viable or of reduced viability. We shall now be able to produce diploid androgenetic urodeles which will be isogenic. If they prove to be viable the problem of haploid lethality would be much nearer its solution. It would show us that the small nuclei and cells are the reason for the feeble viability and not genetic lethal factors. If the viability of these haploids could not be improved by making them diploid then genetic lethal factors would be the cause of early death. A longer survival of diploid androgenetic individuals would also quickly solve the problem of sex determination in urodeles.

Perhaps the production of diploid androgenetic hybrids, which possess the cytoplasm only of one species and the chromosomes from another species, would be of great importance. The study of such abnormal nucleo-cytoplasmic combinations in amphibia has been very difficult because of the early death of the haploids (Baltzer, 1930, 1940, 1949; Hadorn, 1932, 1934, 1937). If the viability of these androgenetic hybrids could be improved, the species specificity of the cytoplasm and also its possible function in the transmission of phenes, or characters (see Hadorn, 1936), could be investigated. Astaurov & Ostriakova-Varshaver's excellent investigation (1957) on the silkworm might then be applied to amphibia.

SUMMARY

1. A simple method has been devised for producing tetraploid newts by means of a 'heat' treatment.

2. The eggs of *T. palmatus* (*helveticus*) and *T. vulgaris* were at different stages, varying from 3 hours to $6\frac{3}{4}$ hours after artificial insemination, exposed to warm water of 34°–38° C. for a duration of 10–12 minutes.

3. A temperature treatment of 35·6°–36·0° C. proved to be the most successful for the production of tetraploid embryos and larvae. Treatment at the stage when the first cleavage furrow closes at the vegetative pole was found to be the only effective one. The eggs contain at this stage two interphase nuclei. The described treatment causes up to 24 per cent. of all treated eggs, or up to 93·7 per cent. of all analysed newts to develop into tetraploids.

4. The probable cytological mechanism which leads to tetraploidy is discussed.

5. Factors which seem to be of importance for the production of tetraploidy in other amphibian species, and some important problems which might be investigated with the help of this method, are mentioned.

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EXPLANATION OF PLATE

Fig. A. Section of 2-cell stage of *T. vulgaris* containing 2 interphase nuclei. ×38.

Fig. B. Medium to late interphase nucleus of 2-cell stage, demonstrating the stage of the nuclei at which heat treatment should be applied. ×455.

Fig. C. Diploid mitosis from tail fin of *T. vulgaris*. ×455.

Fig. D. Tetraploid mitosis from tail fin of *T. vulgaris*. ×455.

Fig. E. Tail tip preparation of a diploid *T. vulgaris*. ×38.

Fig. F. Tail tip preparation of a tetraploid *T. vulgaris*. Note large and few nuclei in this preparation. ×38.

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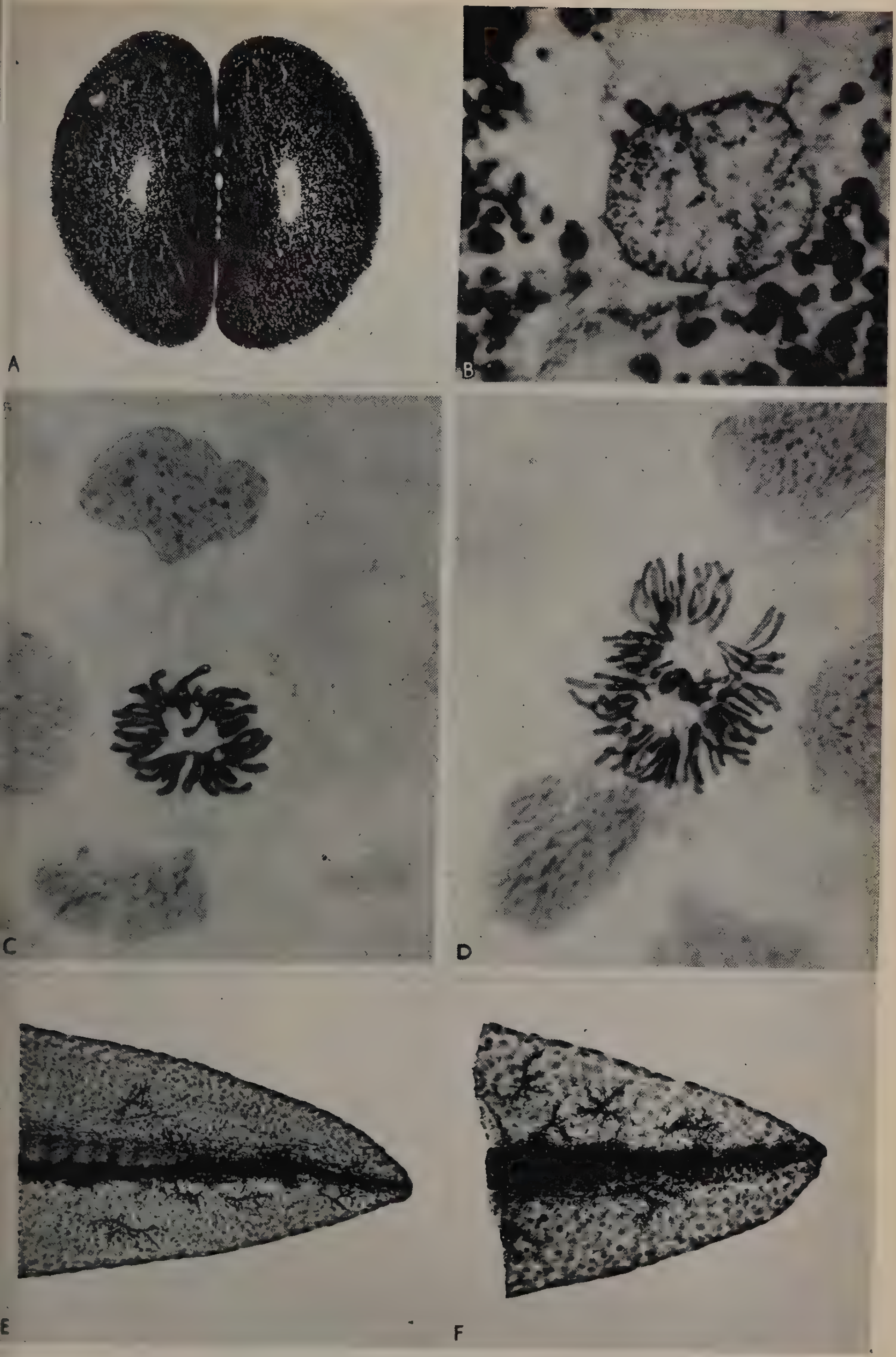

M. FISCHBERG

Intestinal Development

I. Morphogenesis of the Villi and Musculature

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WITH ONE PLATE

INTRODUCTION

THE work of D'Arcy Thompson is a classic example of the insight into morphogenesis that may be gained by a thoughtful consideration of adult form. Even greater insight into the factors underlying morphogenesis might accrue from a detailed study, not only of the adult stage, but also of the intermediate period of development. An attack at this level would, at the very least, yield an account of events whose ordering in time would suggest certain causal sequences and exclude others.

Many organs reveal strikingly simple changes in geometric form during development. These structures provide an opportunity to investigate the role of various factors in the genesis of adult form. We have selected one such organ, the duodenum of the chick embryo, for a series of investigations into the role of mechanical factors in intestinal morphogenesis.

Previous work on chick intestinal development concentrated predominantly on the very early stages when the presumptive gut areas are being blocked out and when initial morphogenesis is in progress (cf. Rudnick, 1944, 1952; Bellairs, 1953 *a*, *b*, 1955). Less attention has been devoted to later stages of development during which the intestine of the chick embryo assumes adult function and form. Some of the functional aspects have been investigated by Moog and her collaborators (Moog, 1950; Moog & Richardson, 1955; Richardson, Berkowitz, & Moog, 1955). Calhoun (1933) cites work dealing with the development of form in her report on the gross morphology of the chicken digestive tract. More recently van Alten & Fennell (1957) have reported on some histochemical aspects of later gut development.

Hilton's (1902) account of how the villi form is noteworthy. It describes geometric changes in the form of the developing villi. These highly patterned

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changes in mucosal topography suggested that mechanical factors are among those which mould the intestine.

This study presents a chronological and quantitative description of developmental changes in the size and shape of the villi and of the duodenum as a whole. In addition it follows the histogenesis of the duodenal musculature. Temporal correlations of this data are used to construct a testable hypothesis concerning some of the forces which orient the smooth muscle-coats and which shape the villi.

MATERIALS AND METHODS

The chick embryos and unfed hatched chicks used were from a Cornish–Rock cross. The eggs were incubated at 37·5° ± 0·5° C., in a forced draft incubator. All ages are given as days following the onset of incubation. In the first phase of the study a partial description of duodenal maturation during intermediate development was compiled by means of mensuration, by counts of villous elements and by histologic study.

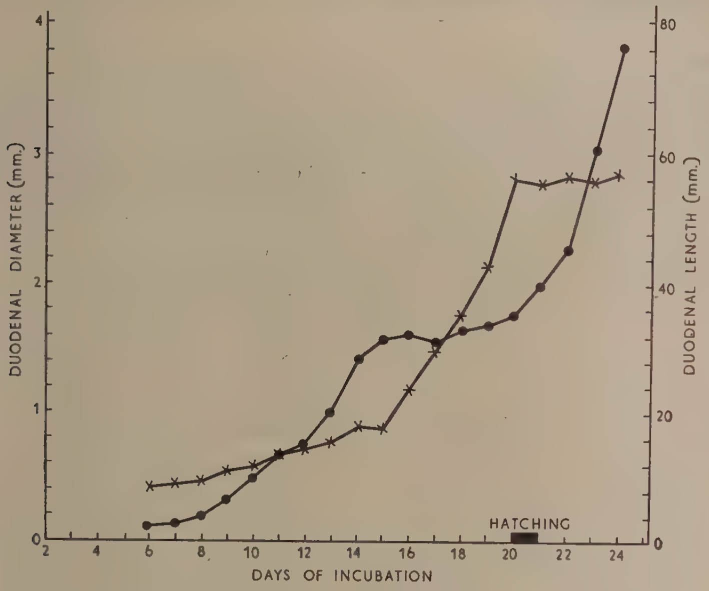
Duodenal length and diameter were measured on a minimum of 10 specimens on each day between the sixth and twenty-fourth. The duodenum was transected at the pylorus and at the duodenal-jejunal flexure. Both of these points are grossly well defined throughout the period studied (Hamilton, 1952). The isolated duodenum was transferred to a glass surface moistened with chick embryo blood. The pancreas was separated from the duodenal loop by cutting the pancreatico–duodenal mesenteries. After properly orienting the duodenum, measurements were made with a calibrated filar ocular micrometer fitted to a dissecting microscope. By slitting the duodenum along its mesenteric border it is possible to lay its wall flat on the glass plate with the mucosal surface uppermost. This permitted measurements and counts of the villi under the dissecting microscope. It also made possible photography of the mucosal surface. For this purpose the specimen was flooded with writing ink which settled in the interstices between the previllous ridges or villi. When such a preparation was transilluminated the previllous ridges and villi stood out sharply in contrast to the ink and were photographed with greater ease.

Histogenetic studies were made with cross and longitudinal sections (5 μ) of Zenker-fixed, paraffin embedded duodenal loops which were collected on each day of development between the fourth and the twenty-fourth. All specimens were stained with hematoxylin and eosin.

RESULTS

Duodenal shape and size

Because the duodenum is a cylinder at all stages during the period investigated, its length and diameter are indices of both size and shape. Graphic representation of these dimensions as functions of age (Text-fig. 1) reveals alternating periods of increase in each dimension. During those periods when there is a rapid increase in length there is no increase in diameter. The converse is also true. Thus, periodic changes in the shape of the duodenum occur as it increases



TEXT-FIG. 1. Duodenal diameter and duodenal length as functions of age. Each point represents the mean of measurements of a minimum of 10 specimens.

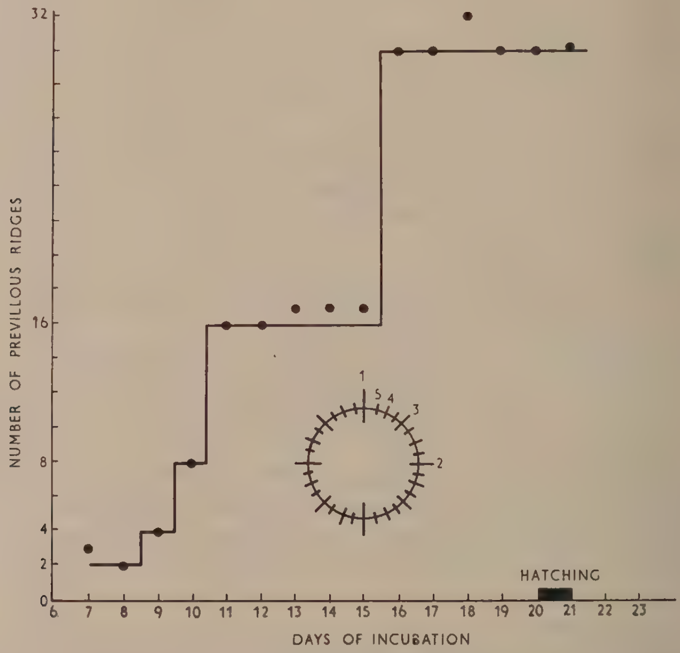
steadily in volume. Between the eighth and the fourteenth days, and again following the twentieth day, the duodenum lengthens both absolutely and relatively. During the intermediate period, from the fourteenth to the twentieth days, an absolute and relative increase in duodenal diameter occurs.

Duodenal musculature

At 4 days the duodenal wall comprises an external cylinder of mesothelium (the serosa), an internal cylinder of pseudostratified epithelium, and an intermediate cylinder of randomly oriented mesenchyme (Plate, figs. A, D). From the fourth to the eighth days the epithelial layer increases in area by cell-division, and the mesenchyme becomes circumferentially oriented. By the eighth day circularly oriented smooth muscle has differentiated in the mesenchyme (Plate, figs. B, E). Following the eighth day, during the first period of duodenal elongation, the undifferentiated mesenchyme remaining outside and inside the layer of circular muscle becomes longitudinally oriented. On the twelfth or thirteenth day longitudinal smooth muscle appears on both sides of the circularis. The smooth muscle which differentiates outside the circularis is the longitudinal layer of the muscularis. The layer inside the circularis is the longitudinal component of the muscularis mucosae (Plate, figs. C, F). Each of the above histogenetic events are observed first at the pyloric end of the duodenum, and progress posteriorly from this point.

The villi

Before the eighth day the mucosal epithelial surface is smooth and there is no evidence of the villi or their precursors. On the seventh or eighth day two longitudinal ridges appear, bulging into the duodenal lumen. Thereafter there is an orderly increase in the number of these previllous ridges in the fashion depicted in Text-fig. 2. Since each successive rank of previllous ridges appears in the grooves between pre-existing ridges (inset, Text-fig. 2) the increase in the number of ridges is stepwise and follows a mathematical progression with a base number of 2 and a factor of 2. That this plan is not followed exactly is due to the occurrence in each duodenum of several anastamoses and bifurcations of the ridges.



TEXT-FIG. 2. The number of previllous ridges as a function of age. Each point represents the mean of counts of a minimum of 10 specimens. The inset represents the circumference of cross-section of the intestine and illustrates the sequence of appearance and the relative positions of the previllous ridges.

Each newly formed rank of ridges is at first more primitive than the previously formed ranks, but it very quickly catches up with its predecessors.

The form of the previllous ridges changes progressively. The first major change is initiated on about the twelfth day when the hitherto straight ridges begin to assume a 'zigzag' form (Plate, figs. G, H). The zigzags are at first irregular, but very rapidly develop a highly regular geometry, and the mucosal surface assumes a herringbone pattern. By 16 days the zigzags have become indented periodically and the definitive villi appear in a manner which preserves the zigzag pattern. At 18 or 19 days the villi elongate rapidly and the pattern, though still in existence, is obscured (Plate, fig. I).

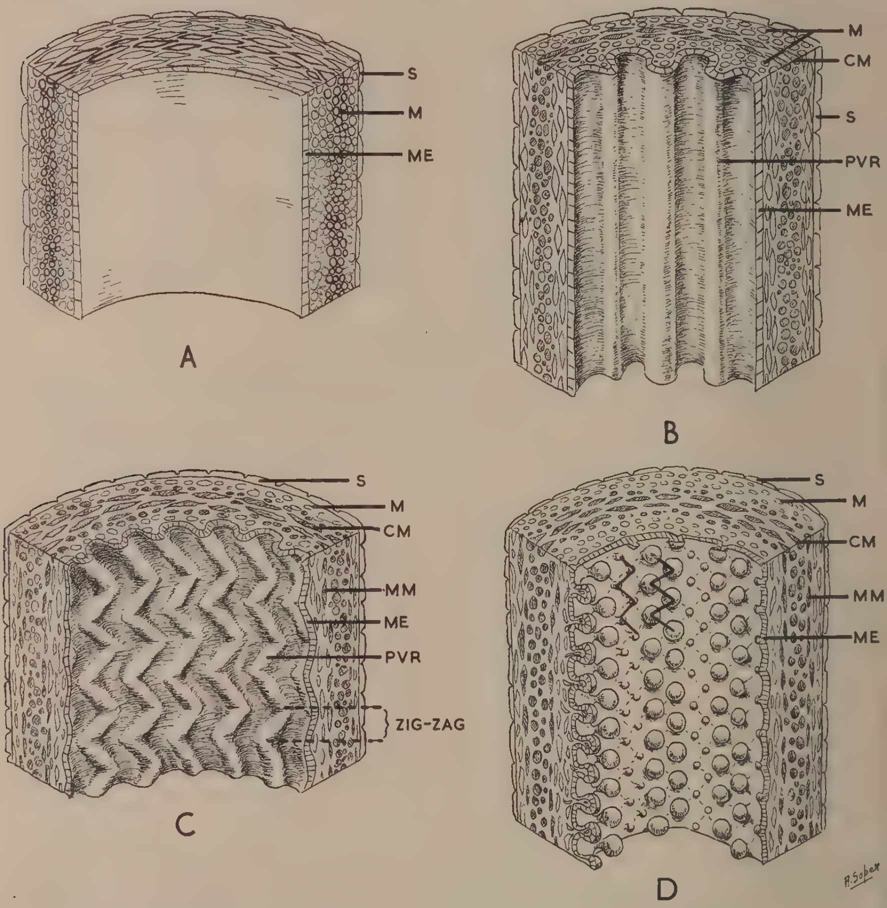
The series of changes described here commence at the pyloric end of the duodenum and progress posteriorly in waves. They are correlated spatially as well as temporally with events in the differentiation of the muscular coats. The significance of this will be treated in the discussion.

DISCUSSION

Correlation of the data on a developmental time-scale leads to an integrated, if incomplete, picture of late duodenal morphogenesis in the chick. The sequence of events which results from such a temporal correlation is based exclusively on observations of normal development. It is, of course, tempting to look on it as a causal sequence, and to assume that each event is a necessary precondition for those which succeed it. Such an interpretation is, at present, hypothetical and must stand or fall on the basis of critical experimental evidence. The available data make it possible to formulate several hypotheses concerning intestinal morphogenesis that are susceptible of experimental test. These are embodied in the following interpretation of the facts (Text-fig. 3).

Between the fourth and the eighth days the mucosal epithelium expands by mitosis. Concomittantly the duodenal mesenchyme becomes circumferentially oriented. It is to be expected that mesenchyme will orient selectively at right angles to a compression line and parallel to a stress line when it is mechanically distorted since it consists of a spongework created by the adhesion of the processes of adjacent cells. It is suggested that the circumferential alignment of the long axes of the mesenchymal cells depends, *inter alia*, upon mechanical factors generated by a relative radial expansion of the duodenum during this period. It is not presently known how much of a contribution is made to this process by mitotic expansion of the epithelial sheet.

On the eighth day a portion of this circularly oriented mesenchyme differentiates into smooth muscle. The orientation of the premuscle mesenchyme seems to determine the orientation of the muscle fibres. This layer comprises the circular portions of the muscularis and muscularis mucosae, which are extensively fused in this species. Contraction of this layer is accompanied, during the ensuing period by two events of significance. Firstly, seemingly as a result of the radial compression imposed by the contracting muscle, the mucosal epithelium becomes thrown into longitudinal folds. These folds are the first rank of previllous ridges. Successive ranks appear in the troughs between previously existing ridges. As a consequence the ridges increase in number by a geometric progression. Secondly, the onset of contraction in the smooth muscle restricts radial expansion of the duodenum and initiates a period of relative elongation. What role this phenomenon plays in the coiling and rotation of the intestine is not yet known. Presumably as a result of the mechanical distortion attending elongation, the undifferentiated mesenchyme on both sides of the circular smooth muscle



Text-fig. 3. Stereodiagrams of the duodenal wall representing different ages: A, 6 days; B, 9 days; C, 13 days; D, 18 days. CM, circularly oriented smooth muscle; M, mesenchyme; ME, mucosal epithelium; MM, longitudinally oriented smooth muscle; PVR, previllous ridge; S, serosal epithelium.

becomes oriented longitudinally. On about the twelfth day the longitudinal components of the muscularis and muscularis mucosae differentiate within this oriented mesenchyme. As these layers contract, duodenal elongation is halted and a period of relative shortening ensues. During this phase the previllous ridges become thrown into zigzags, as though they had been subjected to longitudinal compression. At this stage properly oriented sections reveal what appear to be bifurcating villi. In reality no definitive villi exist at this time, and the illusion of branching is created when zigzags are cut obliquely. At 17 or 18 days periodic indentations appear along the ridges near the points of flexure and the ridges break up into the definitive villi which elongate rapidly.

While detailed data are available only for the duodenum of the chick, casual inspection suggests that the remainder of the intestine follows the same developmental sequence. How far the details can be generalized for other vertebrate species must be determined by additional study. That variations in the sequence do exist is suggested by the existence of straight ridges (turtle) and zigzag ridges (perch, *Amphiuma*, alligator, &c.) instead of villi in the mucosal surface of the adult intestine (Hilton, 1902). This and other observed variations in adult mucosal surface form must result from arrest of or departure from the sequence described here.

It has been suggested that mechanical distortion of the immature duodenum orients premuscle mesenchyme and that this determines the orientation of the first layer of smooth muscle to differentiate. Further, contraction of this first layer of muscle seems to orient residual mesenchyme at right angles to itself and thereby determines the orientation of subsequently differentiating smooth muscle-coats. These principles are offered as a tentative explanation for the fact that hollow viscera such as the ureter, urinary bladder, and fallopian tubes are commonly invested with layers of muscle whose axes bear an angular relationship to each other. In addition, the sculpturing of the inner surfaces of organs like the mammalian fallopian tubes may be dependent in part on the time of differentiation of and the orientation of their smooth muscle-coats.

SUMMARY

A description of chick duodenal morphogenesis is given. It is based on (*a*) quantitative measures of the change in size and shape of the duodenum, (*b*) a histogenetic study of the duodenal musculature, and (*c*) a quantitative description of changes in size, shape, and number of the duodenal villi throughout their development.

Correlation of these data on a developmental time-scale has led to the following hypotheses:

1. Mesenchymal cells are oriented by mechanical forces acting to distort a tissue.

2. The orientation of smooth muscle is determined by the orientation of the premuscle mesenchyme in which it differentiates.

3. In the wall of a hollow viscus such as the duodenum the contraction of the first layer of muscle to form will orient the remaining mesenchymal cells at right angles to itself, thus determining the orientation of the muscle-layers which subsequently differentiate.

4. The duodenal villi are shaped by mechanical forces which act on the mucosa and which arise from epithelial expansion, on the one hand, and muscular contraction, on the other.

ACKNOWLEDGEMENTS

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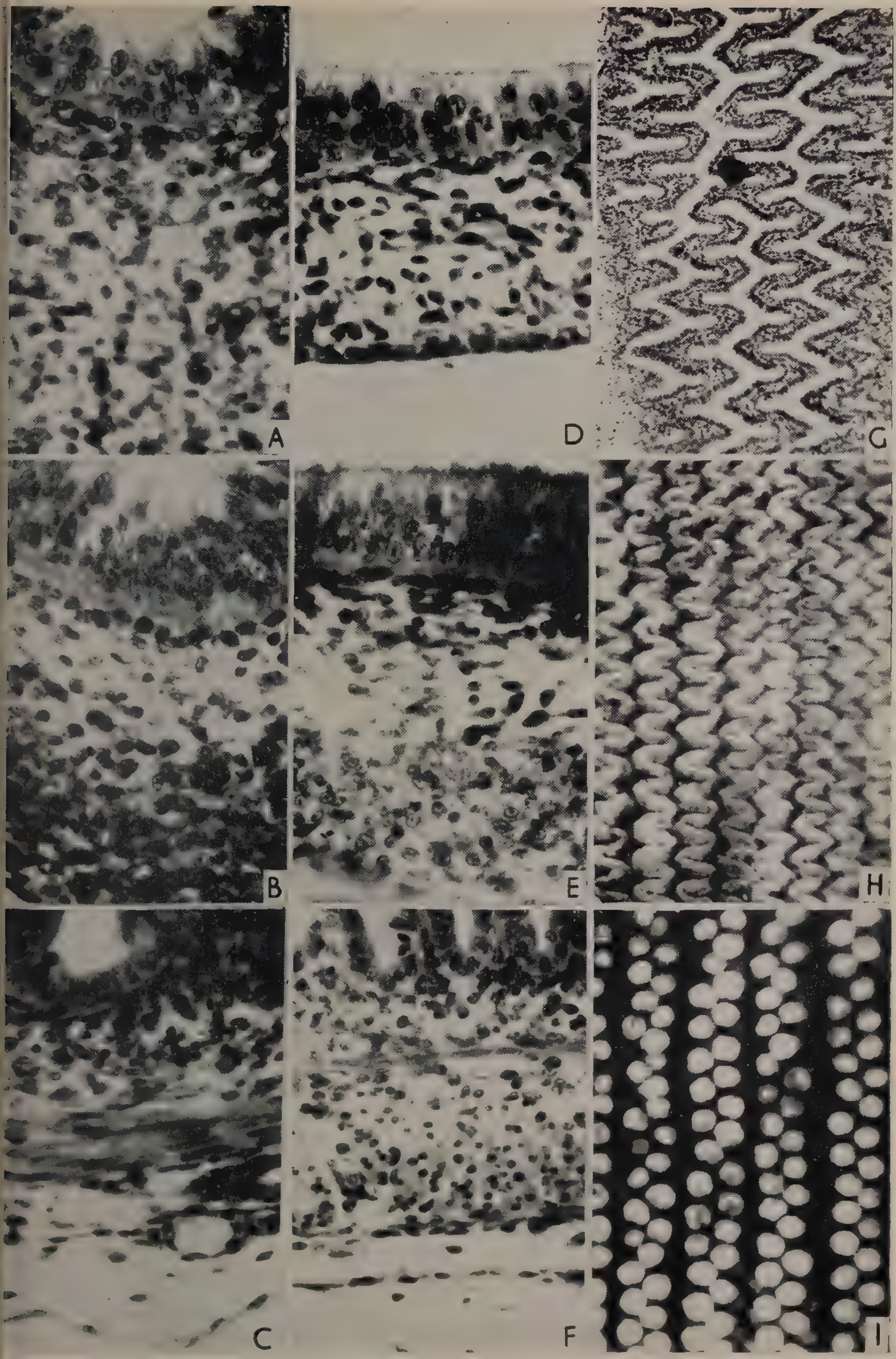
EXPLANATION OF PLATE

FIGS. A, B, C. Portions of cross-sections of the chick embryo duodenum: A, 5 days; B, 8 days; C, 15 days. × 500.

FIGS. D, E, F. Portions of longitudinal sections of the chick embryo duodenum: D, 5 days; E, 8 days; F, 15 days. × 500.



A. J. COULOMBRE *and* J. L. COULOMBRE

FIG. G. Longitudinal section of a 14-day chick embryo duodenum tangential to the mucosa. The zigzags are cut near their bases, and a point of bifurcation is in evidence. × 100.

FIGS. H, I. Fresh, flat whole mounts of duodenal wall. The preparations were flooded with ink and photographed by transillumination: H, 14 days; I, 19 days. × 60.

(Manuscript received 14:i:58)

The Effect of Sodium Thiocyanate on Isolates of the Presumptive Ectoderm and Medio-ventral Marginal Zone of *Triturus* Gastrulae

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As shown by Holtfreter (1944, 1945, 1947), the isolated presumptive ectoderm of *Triturus torosus* develops into a mass of epidermal cells when cultured in a physiological medium. However, if it is treated with injurious media such as acid, alkaline, or Ca-free solutions, or alcohol, and then cultured in a physiological medium, it differentiates neural structures and mesenchyme. These observations were confirmed in experiments made with the ectoderm of *T. pyrrhogaster* (Yamada, 1950; Karasaki, 1957*a*) except for the following points: treatment with Ca-free media or with alcohol does not cause neural or mesenchymal differentiation in the ectoderm of this species, although these treatments lead to a disaggregation of the ectoderm just as with acid and alkali (Karasaki, 1957*a*). For the ectoderm of *T. pyrrhogaster* ammonia was found most effective in producing neural structures. Even differentiation of the mesoderm is altered by treating with ammonia: an isolate of the ventral marginal zone of *T. pyrrhogaster*, with an inherent tendency to form blood-island and mesothelium, differentiate notochord and muscle after a treatment with ammonia (Yamada, 1950). It was emphasized by Yamada that ammonia shifts the mode of differentiation of the isolated ectoderm and mesoderm from ventral towards dorsal. Such effects were designated as dorsalization. Similarly it was demonstrated by Kawakami & Okano (1955) that lactic acid is capable of dorsalizing the presumptive ectoderm and ventral marginal zone.

Ranzi & Tamini (1940) studied the morphogenetic effects of thiocyanate on the ventral part of the early gastrula of *Amblystoma tigrinum* and found enhanced frequency of neural structures. However, no clear effect was observed on the differentiation of mesodermal tissues. According to Ranzi & Tamini (1939) and Ranzi (1945) treatment of embryos of *Rana esculenta* with thiocyanate leads to an increase of the notochordal rudiment. The present writer observed a twisting of the notochord after a treatment of the embryos of *T. pyrrhogaster* with thiocyanate but could not confirm any increase in the total

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cell number and total volume of the notochord. In spite of these negative results it might be possible to obtain a dorsalizing effect of the agent on the mesoderm by the method employed by Yamada for demonstrating the morphogenetic effects of ammonia, because it enables a better penetration of the agent into the cell interior. Hence in the present experiments thiocyanate was tested for its morphogenetic effects on the isolated presumptive ectoderm and medio-ventral sector of the marginal zone of the early gastrulae of *T. pyrrhogaster*.

MATERIAL AND METHOD

The solution of sodium thiocyanate was prepared immediately before use by adding 0·3 c.c. of 1 M sodium thiocyanate to 9 c.c. of Holtfreter's solution. Its pH was adjusted to 7·2 with sodium bicarbonate. A piece of the presumptive ectoderm or of the medio-ventral marginal zone with the narrow strip of the ectoderm directly overlying it was isolated with a glass needle, carefully avoiding the inclusion of the cells of the adjacent territories, and subjected to the thiocyanate-Holtfreter's solution for 5 to 8 minutes. The isolates were then transferred to Holtfreter's solution and cultured at 20°–23° C. for 9 to 10 days. The operation and cultivation were carried out under sterile conditions.

EXPERIMENTS

Experiments on the presumptive ectoderm

(*a*) *Untreated isolates.* All 29 explants developed into atypical epidermis.

(*b*) *Thiocyanate-treated isolates.* A piece of the presumptive ectoderm was isolated in Holtfreter's solution, and transferred to thiocyanate-Holtfreter's solution. Many of the isolates curled so as to expose their originally internal surface outward. After several minutes, mutual adhesiveness of cells apparently lessened and a number of peripheral cells were released from the isolates. Then the latter were returned to Holtfreter's solution, in which the cells regained their

TABLE 1

Structures differentiated in thiocyanate-treated isolates of presumptive ectoderm

Available explants	85	
Explants with structures other than epidermis	61	72%
Explants with neural structures	51	60%
Explants with: Eye	12 (14)	14%
Fore-brain	13 (13)	15%
Nose	7 (11)	8%
Lens	8 (10)	9%
Pigment vesicle	6 (6)	7%
Neural vesicle	13 (13)	15%
Neural mass	37	44%
Mesenchyme	10	12%
Melanophores	11	26%

In parentheses: Actual number of differentiated structures.

adhesiveness and solid aggregates were formed. These aggregates were transferred to a further dish of Holtfreter's solution and cultured. The surface of the aggregate seemed to be occupied by the originally internal surface of the ectoderm. The results of histological observation, shown in Table 1, indicate a considerable number of dorsalized isolates. In 22 of 61 dorsalized isolates archencephalic structures could be identified. The pigment vesicle occurred either isolated in the epidermis or attached to the brain-type structure. Mesenchyme and melanophores differentiated in the direct vicinity of the neural tissue.

Experiments on the medio-ventral marginal zone

(*a*) *Untreated isolates.* The medio-ventral marginal zone was isolated and cultured in Holtfreter's solution. As summarized in Table 2, blood-island, blood-vessel, and mesothelium, which roughly correspond to the presumptive significance of the isolates, were obtained as the main differentiation. The nephric tubules formed within an endodermal mass or in the mesenchyme were short and poorly differentiated. A group of myoblast-like cells without myofibrils was obtained in two cases. In no case was notochordal tissue observed.

TABLE 2

Differentiation of the isolated medio-ventral marginal zone treated or non-treated with thiocyanate

		Control	*Treated explants*
Available explants		39	89
Explants with:	Blood-island	27 (69%)	36 (40%)
	Mesothelium	13 (33%)	21 (24%)
	Blood-vessel	27 (69%)	39 (44%)
	Mesenchyme	24 (62%)	56 (66%)
	Melanophores	0	17 (19%)
	Nephric tubule	10 (26%)	39 (44%)
	Myoblast	2 (5%)	13 (15%)
	Muscle	0	25 (28%)
	Notochord	0	37 (42%)
	Neural tissue	0	10 (11%)
	Ear vesicle	0	2 (2%)

(*b*) *Thiocyanate-treated isolates.* The isolated medio-ventral marginal zone was treated with thiocyanate-Holtfreter's solution until disintegration of the isolate was indicated, and then transferred to Holtfreter's solution, where most of the cells reaggregated. Peripheral cells of the isolates were lost during and after treatment. In this case curling of the isolate so as to turn its originally internal surface outwards was insignificant, probably because of the thickness of the isolate. The results obtained in this series are summarized in Table 2.

As compared with the control series the occurrence of notochord and muscle was noticeable. A well-formed notochord provided with a sheath was found in only 3 explants out of 37. The remaining notochordal tissue was represented by an unorganized mass of vacuolated cells scattered among the myoblasts. Very often the notochordal tissue was accompanied by muscle-cells with myofibrils, or by neural tissue situated on the outer surface of the explant. The structure like an ear vesicle was formed in the vicinity of an elongated notochord. No archencephalic structure was observed. The nephric tubules were much more advanced in differentiation than those of the control series. Although the frequency of blood-islands was only moderately reduced (Table 2), a decisive reduction in the number of blood-cells in each blood-island was apparent, when the present series was compared with its control.

DISCUSSION

The results obtained in the series of experiments on presumptive ectoderm clearly indicate that thiocyanate affects development of the isolated ectoderm in such a way that unorganized neural tissues and archencephalic structures, including mesenchyme and melanophores, are differentiated beside epidermal cells which alone were formed in the control series. The series of experiments on the medio-ventral marginal zone demonstrates that differentiation of notochord and muscle is induced by thiocyanate in the ventral mesoderm, which has the inherent tendency to form ventral structures of the trunk mesoderm such as blood-island, blood-vessels, and mesothelium. Thus for both the ectoderm and ventral mesoderm the effects of thiocyanate-treatment can be designated as dorsalization. The results are closely comparable to those obtained with ammonia (Yamada, 1950), lactic acid (Kawakami & Okano, 1955), and urea (Leone, 1952; Karasaki, 1957*a*).

The neural tissue formed in the thiocyanate-treated mesoderm has probably been derived from the ectodermal component of the isolate. As this component must also have been exposed to thiocyanate, it would have been dorsalized as in the pure ectodermal isolates. However, the fact that ear vesicles, which were never formed in the ectoderm series, appeared in the mesoderm series, needs a special interpretation. In view of their occurrence in the vicinity of the well-formed notochord, it is probable that they were induced from the ectodermal component by the mesoderm dorsalized in its turn by thiocyanate. It may be added that induction of the ear vesicle by the presumptive notochord has been well established (Borghese, 1943), while the capacity to induce is apparently lacking in the ventral mesoderm. That the ventral mesoderm dorsalized by a chemical stimulus is able to induce neural structures has been demonstrated by putting the presumptive ectoderm on an ammonia-treated piece of the ventral mesoderm (Yamada, unpublished). Thus subcytolytic treatment not only changes the mode of differentiation of the ventral marginal zone, but evokes in the latter an inductive capacity comparable to that of the normal organizer.

As stated in the introduction, treatment of the whole embryo of *T. pyrrhogaster* with thiocyanate causes a severe twisting of the notochord, which, however, is not accompanied by an increase in the volume or cell number of the tissue

(Ôgi, 1955, 1957). The difference between those results and the present ones may be due to a difference in the extent of penetration of the substance into the cell interior.

Since there is an antagonistic effect of thiocyanate and lithium on development of the sea-urchin, it is interesting to note that in amphibian embryos the two agents also have antagonistic influences: it is well known that lithium suppresses the formation of the notochord of amphibian embryos, which is enhanced by thiocyanate. Furthermore, the dorsalizing effect of thiocyanate as demonstrated in the present experiments is more or less suppressed by lithium (Okano, 1956; Karasaki, 1957*a*). According to Ranzi (1952) the antagonism is reflected in the effect of both agents on viscosity of various protein solutions. Data of Karasaki (1957*b*) suggest a structural change of the cell as the basis of dorsalization caused by subcytolytic stimuli. This change may be reduced by lithium.

SUMMARY

1. Isolates from the presumptive ectoderm and the medio-ventral marginal zone of the early gastrula of *T. pyrrhogaster* were briefly treated with sodium thiocyanate, and cultured in Holtfreter's solution.

2. The treated isolates of the presumptive ectoderm differentiated archencephalic structures, unorganized neural structures, mesenchyme, and melanophores; whereas its control isolates only formed atypical epidermis.

3. The non-treated isolates of the medio-ventral marginal zone differentiated blood-island, blood-vessel, mesothelium, mesenchyme, and nephric tubule; while the treated isolates of this zone gave rise to notochord and muscle.

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Experiments on the Diffusibility of the Amphibian Evocator

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WITH ONE PLATE

INTRODUCTION

THE discovery by Bautzmann, Holtfreter, Spemann, & Mangold (1932) that amphibian organizer material can still cause an induction after being killed gave rise naturally to the hypothesis that the active principle is a chemical substance which passes from the evocating graft into the reacting tissues. It has, however, remained very difficult to demonstrate that any such passage of material occurs. In this first joint communication, Mangold claimed that when pieces of living blastopore lip are left for some time in contact with an agar jel, the latter becomes inducing, presumably owing to the diffusion into it of an active substance. It has, however, not proved possible to repeat this observation. Most later authors have been impressed with the fact that induction nearly invariably fails if an organizer graft is not in immediate cell-to-cell contact with the reactive tissues, and have concluded that the active principle cannot diffuse through watery solutions. Holtfreter (1933) further showed that induction is prevented if a thin sheet of vitelline membrane is placed between graft and host tissues. Since the permeability of the vitelline membrane is not fully known, it is impossible to draw precise conclusions from this, but it would certainly seem to indicate that the active evocator is not an easily diffusible small molecule.

More recently, several reports have appeared which suggested that the diffusibility of the evocator through membranes might be worth re-investigation. Brachet & de Scoeux (1949) observed induction through a membrane with average pore size of 3·4 μ, but Brachet (1950) later concluded that this was probably due to an unspecific irritation of the reacting cells by the membrane itself. On the other hand, several workers with adult inducers have presented evidence which is suggestive of a diffusibility of active evocating substances. Holtfreter (1955) reported cases of induction by kidney tissue which was coated with agar. Several of the investigators who are attempting to analyse the nature of the heterologous inducers have suggested that compounds such as proteins, nucleoproteins, or even nucleotides may be involved (see the review by von Woell-

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warth, 1956), and these might well be diffusible. Again, induction is known to be possible with liquid 'embryo extract' and solutions of certain carcinogenic compounds. But perhaps the most pressing reasons for re-opening the question arise from the recent works of Niu and Twitty (1953) and Grobstein (1954). The former have shown that if organizer material from an amphibian gastrula is allowed to age for some days in a suitable culture medium, it gives out into solution a substance or substances which are capable of inducing the formation of neural and other tissues in fragments of competent ectoderm. It does not follow that similar substances are emitted from organizer tissue at the time at which it is normally active in the young gastrula, but that possibility certainly exists. Grobstein demonstrated a clear-cut action of mesenchyme on epithelium from which it was separated by a porous filter. The materials used were of the mouse; and the 'inductive' action was perhaps more in the nature of a morphogenetic stimulus than the determination of a particular histogenetic trend; but again the results would seem to make it desirable to re-examine the possibility that the active principle of the primary amphibian evocator might be diffusible through a suitable membrane. Three types of experiment have been made with this aim in view, Gradocol membranes (obtained from the Wright-Fleming Institute, St. Mary's Hospital, London) being placed between the inducing and reacting tissues in various arrangements.

MATERIALS AND METHODS

Sterile conditions were maintained during and after the operations. Full strength Holtfreter solution, buffered at pH 7 (Deuchar, 1953), with 15,000 i.u./l. of both penicillin and streptomycin was used. Operated specimens, when completely healed, were transferred to 1/10 Holtfreter's solution containing 0·1 g./litre of sulphadiazine. Embryos were thoroughly washed with full strength Holtfreter's solution before the operations.

Operations were performed with fine tungsten needles, and the operating dishes were floored with 2 per cent. agar in which a small groove was made to hold the embryos in position during the operations. On occasions, thin glass bridges made out of coverslips of 0·15 mm. thickness were used to assist healing.

Embryos were fixed either with Smith or with Bouin, dehydrated through alcohols, and cleared in terpineol. They were embedded in paraffin, sectioned at 10 μ, and stained with celestine blue eosin.

Series A

The membrane had an average pore diameter (A.P.D.) of 4 mμ, and thickness 0·031 mm. Presumptive neural plate ectoderm of early crescent gastrulae of axolotl was cut near the region of invagination. It was lifted up gently and any cells which remained loosely attached to the inner layer removed carefully without injuring the tissue. A small rectangular piece of membrane was placed between the invaginating mesodermal sheet and the presumptive neural plate.

The presumptive neural plate was brought back to its original position. The embryos were left undisturbed till they healed completely, and then transferred to 1/10 Holtfreter and allowed to develop to the required stages. Six specimens were studied in this series.

Series B

The same membrane was used as in the series A, the experimental material being early gastrulae and early neural plate stages of *Xenopus laevis*. A square piece was removed from an early neural plate without damaging the underlying mesodermal sheet. Presumptive ectoderm of size similar to that of the tissue removed was excised from an early gastrula and grafted over the host with the membrane in between. After healing, the embryos were transferred to 1/10 Holtfreter and reared until the muscular response stage.

Controls were made exactly in the same way except that no membrane was inserted. Eight experimental and six control specimens were studied.

Series C

The membranes had A.P.D. 4 mμ, and thickness 0·031 mm.; and A.P.D. 1·45 μ, and thickness 0·164 mm. Presumptive ectodermal parts were excised from early gastrulae of *Triturus alpestris* and sandwiches were made with early organizer and a small piece of membrane. The membrane was large enough to shield a part of the ectoderm completely from the organizer. The inner layers of the two pieces of ectoderm faced each other. These sandwiches were reared at room temperature (18°–20° C.) for at least 3 days. During this period the solution was changed once. Only healthy specimens were preserved. In this series twenty-one experimental and twenty-two control specimens were studied.

RESULTS

Series A and B showed that the formation of the neural tube can be prevented by the interposition of a membrane between the axial mesoderm and the overlying presumptive neural plate ectoderm. It can be seen in the Plate, fig. 1, that each somite on either side has induced a separate neural tube from the overlying ectoderm in contact with it. These induced tubes are not well differentiated, but the nuclear orientations of the induced tubes (and of the somites) are clear. In another case (Plate, fig. 2) the membrane is a little displaced from its original position, but still separates the mesoderm and ectoderm.

In the control embryos of Series B the axial mesoderm, even as late as the early neural plate stage, induced a neural tube from the grafted presumptive ectoderm (Plate, fig. 3). The induced neural tube is more or less perfect in shape and in differentiation; it is lodged between the somites, in close contact with the notochord. Its development seems to depend mostly upon the chorda and, to a lesser extent, upon the somites.

When a membrane was placed beneath the grafted piece of ectoderm, the part lying over the membrane did not react (Plate, fig. 4). In this figure the neural tube-like structure induced laterally to the membrane on one side is more advanced in its differentiation and development than that on the other side, and a part of it is still in contact with the chorda as well as somite; the other, in contact only with somite, shows only a neural palisade. The same picture is repeated in another specimen. Here there is again an asymmetry between the two small neural elements induced laterally to the membrane, although the larger element is quite separated from the chorda. Again, the part of the grafted piece of ectoderm lying over the membrane has completely failed to react (Plate, fig. 5).

In series C, the controls had induced neural structures which were better than those of the sandwiches containing membranes. With both the thick and the thin membranes, the induced neural palisade lies only on the side next to the organizer (Plate, fig. 6), and is absent over the membrane.

In all these three series no cytoplasmic connexion across the membrane could be observed.

DISCUSSION

The experiments reported above have not revealed any evidence of the passage of inducing influences through micropore membranes, and thus tend to confirm the older impression of the indiffusibility of the natural primary evocator through spaces filled with water or tissue fluids. It may be pointed out, however, that another explanation of the failure of induction is possible. It may be that the quantity of active substance available from the natural organizer during gastrulation is near the effective minimum. It has often been suggested that the area of the neural plate is limited by the diffusion of the active inducing agent from the central region of the archenteron roof towards the sides, the edges of the plate marking the position in which the concentration falls below the threshold of action (cf. Nieuwkoop *et al.*, 1952; Waddington, 1956). If this were the situation, the evocator might still diffuse through the membrane but fail to attain a high enough concentration at the other side to be effective. One could in this way safeguard the hypothesis that the natural evocator of the gastrular archenteron roof is similar in chemical nature to the active substances which have been extracted from various heterologous inductors. On the other hand, it would certainly be most unwise to neglect the possibility that the inducing action of the archenteron roof depends on a cell-to-cell contact, as Weiss (e.g. 1950) has argued is the case for the lens-inducing action of the optic cup. In view of the negative character of the results reported here, this is perhaps not the occasion to attempt a lengthy discussion of the pros and cons of these alternative possibilities.

SUMMARY

1. Presumptive neural plate ectoderm of an early gastrula, when separated by a porous membrane (average pore diameters, 4 mμ) from the axial mesoderm, failed to differentiate into neural tube either in *Axolotl* or in *Xenopus*.

2. In ectodermal sandwich experiments induction always occurred on the side containing the organizer in *Triturus alpestris* and was prevented by the interposition of a porous membrane, when the latter had an average pore diameter of 4 mμ or 1·45 μ.

3. No cytoplasmic connexion across the membrane could be observed.

4. The lack of induction through the membranes might be due either to a total failure of diffusion of an active substance, or to a failure of a diffusing substance to attain a high enough concentration on the other side of the membrane, or the induction may essentially involve cell-to-cell contact. No decision between these alternatives is reached.

ACKNOWLEDGEMENTS

I express my deep gratitude to Professor C. H. Waddington, F.R.S., for suggesting to me this problem and for his valuable criticism of the manuscript. I am also thankful to the Calcutta University for a T. N. Palit Foreign Scholarship.

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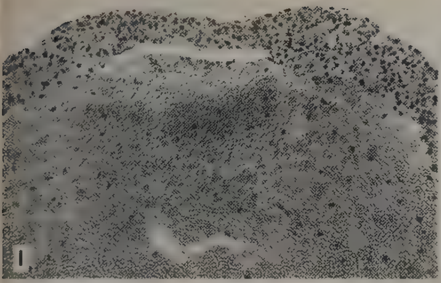
EXPLANATION OF PLATE

Fig. 1. Transverse section through the trunk zone of an *Axolotl* embryo. The membrane (which has been dissolved during histological preparation) completely separates the presumptive neural plate from the chorda. On both sides the somites have induced neural tube-like structures. × 160.

Fig. 2. Transverse section through the trunk zone of an *Axolotl* embryo. The membrane is slightly displaced, but still separates the ectoderm and mesoderm. On either side, somites have induced neural tube-like structures. × 130.

Fig. 3. Transverse section through the trunk zone of a *Xenopus laevis* embryo. No membrane. The neural tube is formed from the grafted piece of presumptive early gastrula ectoderm.

Fig. 4. Transverse section through the trunk zone of a *X. laevis* embryo. The membrane completely separates the grafted piece of presumptive ectoderm from the chorda. × 115.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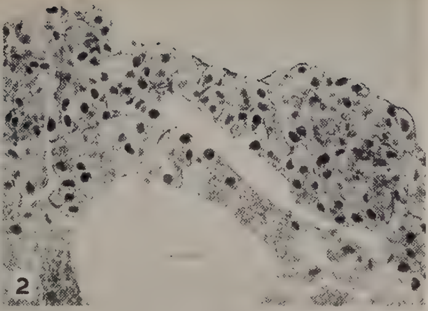
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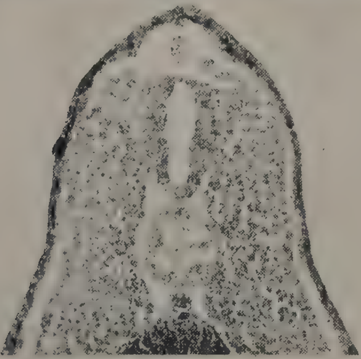
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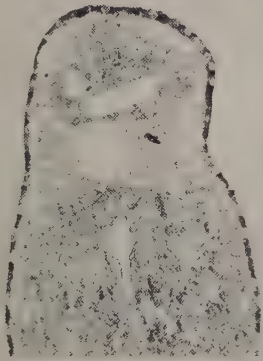
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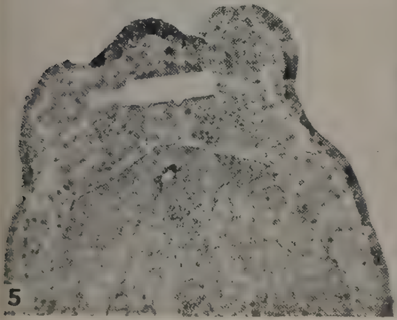
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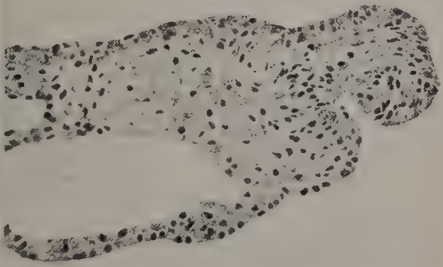
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FIG. 5. Transverse section through the trunk zone of a *Xenopus* embryo. The membrane completely separates the grafted piece of presumptive ectoderm from the chorda. On both sides the induced neural tubes are not well differentiated due to an early fixation. ×60.

FIG. 6. Transverse section of an ectodermal sandwich of *Triturus alpestris*. Part of the ectoderm separated by the membrane is completely non-reactive and the neural palisade is on the side containing the organizer. ×100.

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Genetical Studies on the Skeleton of the Mouse

XXIII. The Development of Brachyury and Anury

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WITH THREE PLATES

INTRODUCTION

FOLLOWING the discovery of the ventral ectodermal ridge of the tail in mouse embryos (Grüneberg, 1956), it became necessary to test its possible influence on tail development in those mutants whose effects on the axial skeleton are traceable to the 10–11-day stage, i.e. to the period when the development of the ventral ectodermal ridge (V.E.R.) is at its height. In the case of vestigial-tail (vt/vt; Grüneberg, 1957), virtual absence of the tail was associated with a marked reduction of the V.E.R. to one-quarter or less of its normal size. In Danforth's short-tail ($Sd/+$ and Sd/Sd; Grüneberg, 1958), on the other hand, reduction or absence of the tail was accompanied by no more than a slight reduction in size of the V.E.R.

The present paper deals with Brachyury ($T/+$ and T/T) and with Anury or taillessness (T/t^6 and T/t^1 respectively). Short-tail or Brachyury is a semi-dominant condition lethal in homozygotes (Dobrovolskaia-Zavadskaia, Kobozieff, & Veretennikoff, 1934; Kobozieff, 1935). The development of $T/+$ and T/T has been studied mainly by Chesley (1935). As discovered by the Parisian investigators and since studied in great detail by Dunn and his collaborators (for a recent summary see Dunn, 1956), there exist many recessive 'alleles' of T which, in T/t^n heterozygotes, lead to taillessness or Anury; $+/t^n$ heterozygotes are phenotypically normal, but the homozygotes of the majority of the t-alleles are early embryonic lethals. In such cases, balanced stocks $T/t \times T/t$ breed true to taillessness with the elimination of the lethal T/T and t/t homozygotes. The development of taillessness in two such stocks, T/t^0 and T/t^1, has been studied by Gluecksohn-Schoenheimer (1938).

The present work was carried out in the first instance to study the V.E.R. in $T/+$ and T/t embryos (T/T embryos entirely lack the posterior trunk region and hence cannot develop a V.E.R.). In the course of this work some, but not all, of the observations of previous investigators have been confirmed; certain

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additional facts have also been discovered. Like Chesley (1935) and Gluecksohn-Schoenheimer (1938), the present author has come to the conclusion that the notochord is probably the structure primarily affected in these genotypes. However, as will be shown in this paper, the pathological process appears to be rather different from that postulated by the earlier authors.

BRACHYURY ($T/+$)

The abnormalities of the axial skeleton of adult $T/+$ mice have been described in some detail by Dobrovolskaia-Zavadskaia and her collaborators. A single fact may here be added. It seems that Brachyury, in common with many other genes in the mouse, affects the number of presacral vertebrae. Alizarin clearance preparations are available of 12 $T/+$ mice and 12 $+/+$ normal litter mates aged 24–33 days; all of these except two $+/+$ mice are males. Two $T/+$ brothers show major pathological changes in the lumbo-sacral region, so that the mode of attachment of the pelvis to the vertebral column cannot legitimately be compared with that in normal mice. If these two animals and their normal brothers are excluded, the data of Table 1 are obtained in which 25½ presacral vertebrae stands for an asymmetrical attachment. Pooling animals with 25 and 25½ presacrals and treating the resulting fourfold table by means of Fisher's 'exact' method, the probability that the difference observed is due to chance alone is just under 0·01. Hence it seems that $T/+$ mice tend to have fewer presacral vertebrae than their $+/+$ sibs, at least on the heterogeneous genetic background of the present stock. Whether the same trend would be found on a different genetic background is, of course, another question.

TABLE 1

Number of presacral vertebrae in $T/+$ and $+/+$ mice

	Number of presacral vertebrae			
	25	25½	26	*Total*
$+/+$	2	1	7	10
$T/+$	8	1	1	10

At the time the embryonic material was collected, the expression of $T/+$ was fairly moderate. Of the twelve animals just mentioned, one had a very short tail with a jumble of a few osseous elements only; one had eight caudal vertebrae; the remaining ten animals had between 18 and 24 caudals with an average of about 21; in the majority of $T/+$ mice, the tail was thus reduced by about one-third as judged by the number of vertebrae and by less as judged by tail length. Otherwise, with the exception of disturbances in the lumbo-sacral region in two of the animals, the axial skeleton cranial to the tail was virtually normal.

The development of Brachyury was mainly studied in embryos from $T/+ \times +/+$ matings. Thirty-nine embryos from 15 litters aged 11–14 days (mean crown–rump lengths 5·1–10·3 mm.) were selected by the external appearance of their tails, and the tails only were sectioned. In embryos of 10½ days or less (Table 2), classification was no longer possible by external features; hence complete serial sections of all the embryos were prepared in litters 16–28 (Bouin fixation; embedding by Peterfi's method; sections of 7·5 μ, except three embryos in litter 16 which were sectioned at 10 μ; haematoxylin and eosin). With the exception of the four embryos whose C.R.L. is given in italics, classification presented no difficulties; if these four embryos are included, there are 42 + / + and 35 *T* / + individuals; if they are excluded, the ratio is 39 : 34. Both values are close to the expected 1:1 ratio. Additional *T* / + embryos were studied in the *T* / + × *T* / + matings discussed in the next section.

The following descriptions will deal mainly with the early stages of the *T* / + anomaly, i.e. with embryos aged 10½ days or less when, in the present material, no regressive processes have yet started in the tail. The pyknotic changes which later lead to the loss of the tail, or part of it, need not be described here, as my own findings are in essential agreement with those of Chesley (1935) and of Gluecksohn-Schoenheimer (1938).

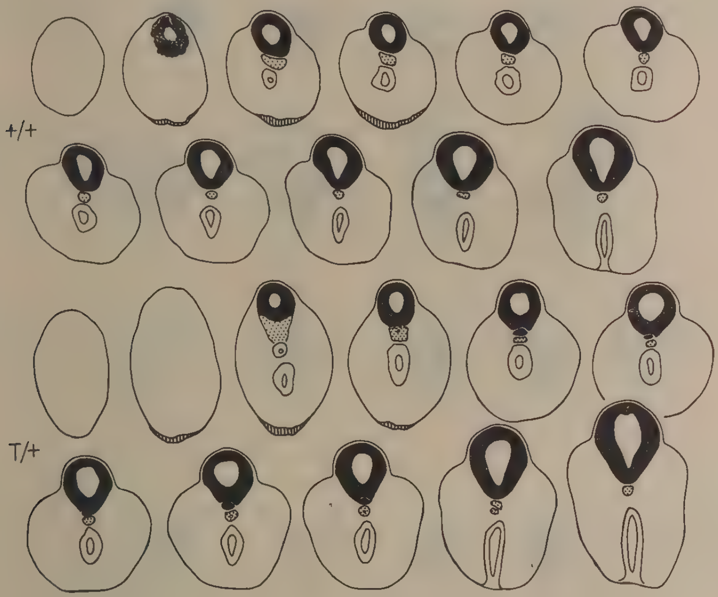
TABLE 2

Serially sectioned embryos from T / + × + / + matings

Litter	*Nominal age*	*Crown-rump length (mm.)*		*Mean C.R.L.*
		+/+	*T*/+	
16	10½	3·9 4·7 4·5	4·4 4·3 4·6	4·4
17	10½	4·7 4·4 4·5 4·3 3·7	4·3 4·6 4·0	4·3
18	10	3·9 4·1 4·0	3·8	4·0
19	10	3·8	4·0 4·0 3·6 4·0	3·9
20	10	3·7 3·7 3·4	3·6 3·5 3·8 4·0 3·6 3·7	3·7
21	9¾	3·4	3·3 3·5 3·6 *3·4*	3·4
22	9½	2·9 2·9 *2·6*	3·1 2·8 2·9 2·9	2·9
23	9½	3·0 3·3 2·9 2·9 *2·8*	2·8 2·4	2·9
24	9½	2·9 2·9 3·1 2·6 3·1	2·7 2·8	2·9
25	9½	3·0 2·5 2·9 2·8	2·6	2·8
26	9½	2·8 2·8 2·7		2·8
27	9½	2·4 2·8 2·8	2·8 2·7	2·7
28	9	2·0 1·8 *1·2*	1·9 1·8 1·7	1·7

The notochord

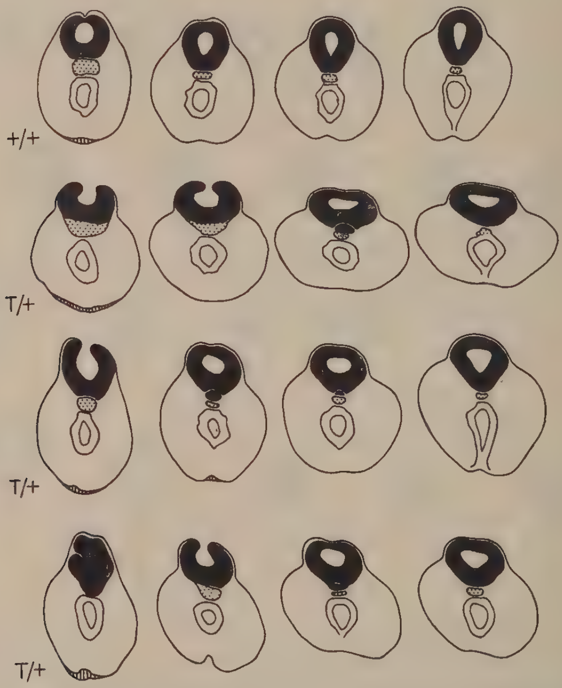
Before turning to a systematic description of the anomalies of the notochord in *T* / + embryos, attention should be drawn to a feature which has not been mentioned by previous investigators. The notochord of *T* / + embryos has, in places, a central lumen. This lumen is usually confined to the tail, but sometimes extends as far forward as the cloacal region. The size of the lumen varies from a pin-point foramen in two or three consecutive sections to a large hole which transforms the notochord into a tube for a distance of 100–200 μ or more. Often, several regions of a notochord show lumina which do not communicate with each other. Large lumina are often surrounded by a single layer of notochordal cells so that the whole structure is reminiscent of an epithelium. Examples of notochordal lumina are shown in Text-figs. 1, 2, and 4 and in Plates 1 and 2, figs. 7, 11, and 14. Lumina have been encountered in *T* / + embryos from all litters listed in Table 2 as well as in older specimens. Of the 35 *T* / + embryos of Table 2, 22 had large lumina, 9 had only small foramina, and in 4 no foramina have been discovered. One of the largest lumina seen occurred in a 9-day embryo of C.R.L. 1·7 mm. in litter 28.



TEXT-FIG. 1. Transverse sections through the tails of a normal embryo (top two rows) and a *T* / + litter mate (bottom two rows), from litter 18 (nominal age 10 days; C.R.L. 4·1 and 3·8 mm. respectively). In each case projection drawings of sections 10, 20, 30, . . ., 110 as counted from the tail tip are shown. In all text-figures neural plate or tube is shown in black, notochord stippled, tail-gut or cloaca in white and the ventral ectodermal ridge of the tail hatched. Drawings made at magnification ×250: final magnification ×62·5.

The following description will be simplified by anticipating some of the results of the analysis. In early stages of development, the notochord of *T* / + embryos tends to be retained by structures from which it should have become separated at an earlier stage (roof of hind-gut and / or cloaca and primitive streak). Later on, the notochord of the tail has a tendency to become incorporated in one of its neighbours (neural tube, or tail-gut, or both). These processes will now be described in more detail by reference to Text-figs. 1–4 and Plates 1–3.

(1) *Separation of the notochord from the primitive streak.* In normal mouse embryos aged 9–$10\frac{1}{2}$ days, the notochord becomes fully separated from the overlying neural plate or neural tube and from the paraxial mesoderm in the neighbourhood of section 38 (of 7·5 μ) as counted from the end of the tail or of the body (Grüneberg, 1958). The growing end of the notochord is a flat plate in 9-day embryos; in $9\frac{1}{2}$–10-day embryos it usually has a rectangular cross-section with rounded corners, and in still older embryos the distal end of the notochord becomes increasingly cylindrical.

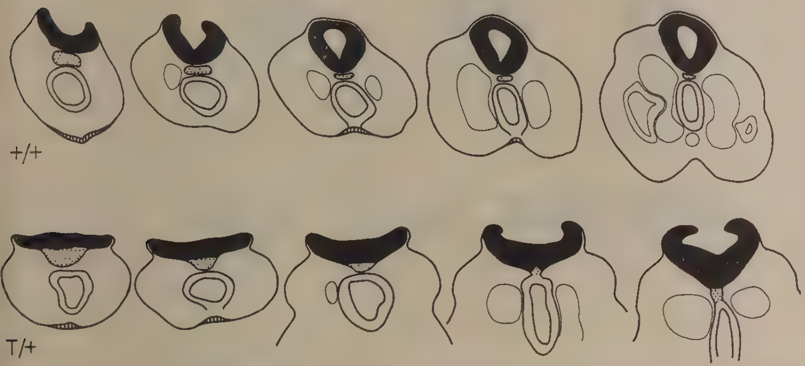


TEXT-FIG. 2. The 30th, 40th, 50th, and 60th sections through the tails of a normal embryo (top) and three $T/+$ litter mates, from litter 21 (nominal age $9\frac{3}{4}$ days; C.R.L. 3·4, 3·3, 3·5, and 3·6 mm. respectively).

In $T/+$ embryos the growing end of the notochord tends to be larger than normal and is often very bulky. Usually its base on the neural tube is much wider than normal while ventrally it tapers towards the tail-gut. Its cross-section thus looks somewhat as if an artificial beard was dangling from the ventral circumference of the neural tube. This configuration is quite typical for $T/+$ embryos and is shown in Text-fig. 1 (section 30) and in Text-fig. 2 (sections 30 and 40 of the first and third $T/+$ embryo). The 'beard' is generally not demarcated from the neural tube by a sharp border, but merely by a zone in which the cells are less closely knit.

In older embryos the separation of the caudal end of the notochord is not much delayed. A tolerable separation has been achieved by section 40 in Text-ig. 1; in two of the $T/+$ embryos of Text-fig. 2 there is no proper separation until section 50, and in the still younger embryo of Text-fig. 3 there is no proper separation as far cranial as section 60.

The massive structure which differentiates out of the primitive streak in $T/+$ embryos seems to contain not only cells destined to form notochord, but also materials with neural affinities. This is shown by the fact that the 'beard' nearly always soon divides into a dorsal mass (which is usually larger) and a ventral structure which forms the notochord proper. The dorsal mass soon gains contact with the ventral aspect of the neural tube. Usually, it first lies in a groove and soon merges more or less completely with the neural tissue; for some distance the arrangement of the nuclei usually indicates that the ventral part of the neural tube was originally separate, but soon the material is so completely incorporated as to be indistinguishable from its surroundings. The process is shown very clearly in Text-fig. 1 where the dorsal moiety is still just detectable in section 90, but has vanished in section 100. It is also seen in Text-fig. 2 where it vanishes in the neural tube between sections 50 and 60 in each instance.

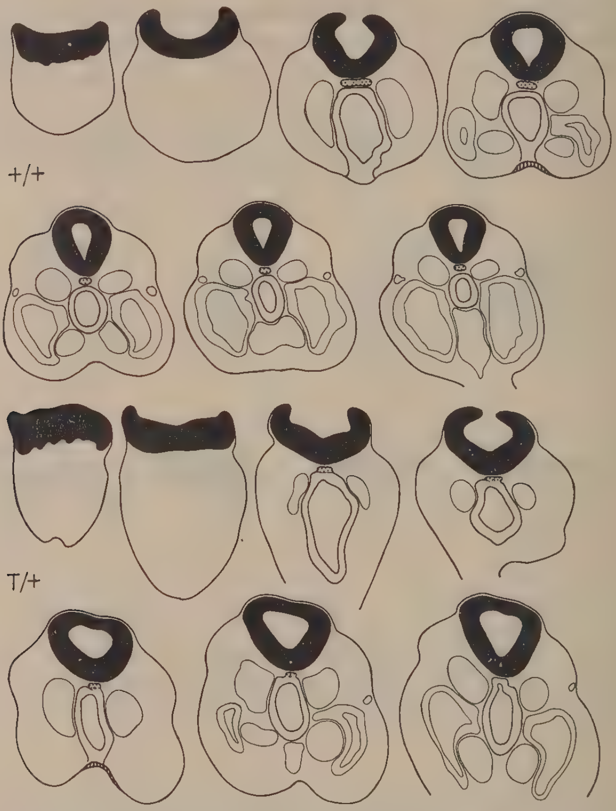


Text-fig. 3. The 30th, 40th, 50th, 60th, and 70th sections through the tail and adjacent cloacal region of a normal embryo (top) and a $T/+$ litter mate, from litter 22 (nominal age 9½ days; C.R.L. 2·9 and 3·1 mm. respectively). In this and the following text-figure the dorsal aortae, the roots of the umbilical artery, and the common umbilical artery are indicated by single contours; the coelom is shown by double contours; in fig. 4 the Wolffian ducts are also shown.

While proximally the dorsal mass is very completely incorporated in the neural tube, the caudal end nearly always remains separate and in fact shows signs of growth. Originally solid, a secondary lumen (which does not communicate with the neural canal) is generally formed; this is preceded by a radial arrangement of the nuclei near the periphery, as shown in Plate 1, fig. 6.

(2) *Branches of the notochord.* Whereas the separations described in the

preceding paragraph seem to do nothing but disencumber the notochord of extraneous matter, branching of the notochord proper is also commonly encountered in *T*/+ embryos. In 10–12½-day embryos, such branches occur mainly in the region of the cloaca and the proximal parts of the tail; two or three independent branches may be found in the same embryo. An example is shown in Text-fig. 1 where the notochord is double in section 100. In this case, the calibre of the notochord does not suggest that it includes extraneous materials which have here been hived off. The smaller ventral branch in this case can be traced through 5–6 sections in a proximal direction where it ends blindly. Of 11 *T*/+ embryos in litters 16–19, 7 showed such branches of the notochord. Most of them are short, but one was traceable through some 35 sections. In all instances the fork is closed



Text-fig. 4. The 10th, 20th, . . ., 70th sections through the posterior end of the body of a normal embryo (top two rows) and a *T*/+ litter mate, from litter 30 (nominal age 9½ days; C.R.L. 2·2 and 3·0 mm. respectively; mean C.R.L. of litter, excluding one *T*/*T* embryo, 2·73 mm.).

caudally; i.e. there must have been a single growing notochord which started to branch secondarily afterwards. Usually the side branch ends blindly, but in some instances the two structures coalesce again proximally. Sometimes the splitting of the notochord happens in such a way that an existing lumen forms part of the plane of separation. In younger $T/+$ embryos (litters 20–22), branches of the notochord are uncommon; no really clear case was encountered among 14 $T/+$ embryos in these litters though there were one or two suspicious regions.

(3) *Retention of the notochord in the roof of the cloaca.* As is well known, in the normal development of mammals and other vertebrates, the notochord for a time is interpolated into the continuity of the roof of the gut. In the mouse (Snell, 1941) it is separated again from the roof of the gut throughout its length by the time the embryo turns from its original position with concave back to its final posture with back convex, i.e. from the age of 9 days onwards. The present author, in previous studies, has never encountered an incomplete separation of the notochord from the roof of the hind-gut or cloaca in turned embryos, normal or carrying the genes for vestigial-tail or Danforth's short-tail.

The situation is different in $T/+$ embryos. It is commonly found that the notochord is more or less completely retained in the roof of the cloaca. The extent of this retention is very variable. In the most extreme cases the retention starts well cranial of the cloaca in the hind-gut; distally it may extend some way into the tail-gut. At the other end of the scale the region of contact may be small, and the notochord may no longer be in the continuity of the wall of the hind-gut or cloaca, but may lie dorsally on top of it as a structure which is firmly attached but recognizably a separate entity.

A typical example is shown in Text-fig. 4. In the normal embryo the notochord is a separate structure in sections 30–70. In the $T/+$ embryo the notochord is firmly glued on to the dorsal roof of the cloaca in sections 30–60, with a small lumen in sections 40–60. Cranial to this region the notochord is so completely incorporated into (or rather, retained in) the hind-gut that, for a long distance, it cannot be recognized as a separate structure. Its presence in the roof of the gut can indeed only be demonstrated by the fact that cranially the notochord can be followed into, and caudally out of, the roof of the gut in serial sections. In this particular case, the lumen of the notochord is continuous with that of the gut; this is unusual; in most $T/+$ embryos the lumina of the notochord do not communicate with other cavities.

A much less extreme case is shown in Text-fig. 3. Here, in section 70, the notochord is in direct contact both with the hind-gut and with the neural plate above; in this region, as also shown clearly in section 60, the notochord is in the process of changing partners: cranially its separation from the gut has been delayed, while caudally the same is happening in relation to materials which dorsally hang together with the neural plate and caudally with the primitive streak. A somewhat similar situation is also shown in the first $T/+$ embryo of Text-fig 2. Here the notochord is still in close contact with the cloaca in section 60, and the change of partners takes place between sections 50 and 60. Altogether, in this embryo relations of varying degrees of intimacy between notochord and cloaca can be traced through some 18 sections (or about 135 μ).

A very clear case is shown in Plate 2, figs. 13–18. In this 10-day embryo (C.R.L. 3·5 mm.) the notochord is caudally completely free of its neighbours; in the section shown in fig. 15, it is dorsally attached to the cloaca with whose wall it merges very completely and for a long stretch later on (or, more accurately, in which it has been retained earlier on), as illustrated in figs. 16–18.

In a little more detail the situation is shown in figs. 19–21 (nominal age 9½ days; C.R.L. 2·8 mm.) and in figs. 22–25, Plate 3 (nominal age 9 days; C.R.L. 1·7 mm.). In these embryos the process of the delayed separation of the notochord from the hind-gut can be seen in a number of more closely spaced sections.

The retention of the notochord in the roof of the hind-gut is very common in young $T/+$ embryos of 9–9½ days. Of 14 such embryos in litters 22–28, 11 showed these delays to a greater or lesser extent. In later stages they are rarely encountered (the specimen shown in Plate 2, figs. 13–18 being rather exceptional in this respect). Obviously, the separation of the notochord from the roof of hind-gut and/or cloaca is ultimately successful in the majority of $T/+$ embryos.

(4) *Secondary incorporation of the notochord into the neural tube or the tail-gut.* The anomalies in the differentiation of the notochord from the primitive streak described under (1) and the retention of the notochord in the roof of the gut described under (3) are essentially delays of normal processes. They tend to occur mainly in young $T/+$ embryos. We now have to add a category of anomalies which is confined to the tail proper and which occurs mainly in embryos aged 10½ days and over. A notochord which has been quite free both dorsally and ventrally can secondarily attach itself to one of its neighbours (or both) and can fuse with them very completely. An example is shown in Plate 1, figs. 1–6. Distally (fig. 1) the notochord is separate; in fig. 2 it starts to fuse with the neural tube, a process which is complete in fig. 3; soon, however, the composite for a short stretch divides into two tubes of roughly equal calibre; the dorsal tube is composed entirely of neural tissue, the ventral one obviously is composite; these two tubes merge again more cranially, and eventually a rather small notochord 'segregates' out in fig. 5.

Another example is given in Plates 1 and 2, figs. 7–12. Here a distally free notochord becomes attached to and later merges with the tail-gut; in fig. 10 there is a conglutination of neural tube, notochord, and tail-gut into one structure; more proximally (fig. 11), the notochord is free, and still more proximally (fig. 12) the notochord is once more integrated in the dorsal wall of the tail-gut for a long stretch. In this case it is not certain whether there are two independent regions in which the notochord has become secondarily incorporated in the tail-gut, or whether the more proximal zone should be interpreted as a persistence of the notochord in the gut-wall as discussed under (3) above. There are a few other cases in which it is not certain whether the distinction here made between (3) and

(4) for descriptive purposes is quite sharp. However, this is probably of no great importance as all the anomalies of the notochord here described seem to spring from the same source.

From the descriptions given above it is clear that $T/+$ embryos possess a notochord from head to tail tip. The notochord may be retained in the dorsal wall of the hind-gut, of the cloaca or of the tail-gut; it may be secondarily incorporated for a distance in the tail-gut, in the neural tube, or in both; or it may hide for a time in continuity with the neural plate. It is, however, always possible to trace its continuity from one end of the embryo to the other in serial sections. If the notochord disappears in one structure it will ultimately re-emerge or segregate out from that structure at some other level. However much it becomes integrated in one of its neighbours, it does not lose its identity as shown by its re-emergence elsewhere.

In a single embryo (the $10\frac{1}{2}$-day-old specimen of Plate 1, figs. 1–6) has the whereabouts of the notochord been lost track of for a stretch in the cloacal region. The difficulty is probably only partly accounted for by the unfavourable plane of sectioning. It may be that in this embryo the notochord may have actually disintegrated for a distance. Whatever the explanation of this particular case may be, it does not seem to invalidate our general conclusion that $T/+$ embryos have a notochord from one end of the body to the other.

It should be pointed out that when the notochord re-emerges from a structure in which it has been retained or secondarily incorporated, it is sometimes thinner in calibre than normal; this suggests that what comes out of such a host structure is not the whole notochord, but that some notochordal material has been retained. Conversely, it sometimes happens that a 'segregating' notochord is thicker than normal; this suggests that the notochord may have taken with it some extraneous material from its host. There are also sometimes changes in the calibre of an unattached notochord, the causes for which are not clear. For instance, in the last $T/+$ embryo of Text-fig. 2, the notochord increases in calibre considerably between sections 50 and 60 for no obvious reason.

Apart from their involvements with the notochord described in the preceding sections, neural tube and hind-gut, cloaca, and tail-gut appear to be quite normal. Possibly an additional anomaly is some slight delay in the closure of the posterior neuropore which is common in $T/+$ embryos. No anomalies of the paraxial mesoderm have been discovered.

The ventral ectodermal ridge of the tail (V.E.R.)

The size of the V.E.R. was measured by the number of $7{\cdot}5\ \mu$ sections in which the structure could be detected. The size in normal embryos (Table 3) is in close agreement with that found in previous series of normal embryos (Grüneberg, 1957, 1958). The size of the V.E.R. is significantly reduced in $T/+$ and particularly in T/t^6 and T/t^1 embryos. As has been pointed out previously, the accuracy of such a count is limited by the fact that the edge (and particularly the proximal edge) of the V.E.R. is not sharp, so that a certain degree of arbitrariness is inevitable; it is believed that in the present series this may have led to some overestimate of the size of the V.E.R. in *T* / + and *T* / *t* embryos. Taking the means of Table 3 at their face value, the V.E.R. of *T* / + embryos is reduced to 74 per cent. and that of the combined *T* / *t* embryos to 69 per cent. of the normal size. Actually, as in many cases, the dorso-ventral thickness of the structure is also clearly reduced, normal and abnormal embryos probably differ rather more in size of the V.E.R. than is suggested by the data of Table 3.

TABLE 3

Number of sections of 7·5 μ thickness in which the V.E.R. can be identified. The means are based on the original (ungrouped) data. The + / + *and T* / + *embryos are from litters 16–21, Table 2*

Sections	*11–15*	*16–20*	*21–25*	*26–30*	*31–35*	*36–40*	*41–45*	*46–50*	*Mean*
+/+	..	..	..	1	2	7	3	3	39·5
T/+	2	2	1	7	4	4	..	1	29·1
T/t^6	..	2	1	1	..	2	..	..	27·2
T/t^1	..	1	1	1	1	1	..	..	27·4

THE *T*/*T* HOMOZYGOTE

According to Chesley (1935), *T* / *T* embryos can be recognized before turning (i.e. when the embryos are still S-shaped with concave back on the 8th day) by the presence of usually paired subepidermal blebs on the back; these blebs disappear on turning without leaving a trace. Later, *T* / *T* embryos can easily be identified by the incomplete development of the posterior end of the body. As discovered by Gluecksohn-Schoenheimer (1944), the allantois of *T* / *T* embryos fails to make contact with the ectoplacental cone, with the result that the umbilical vessels do not develop. *T* / *T* embryos die towards the end of the 10th day and are subsequently absorbed.

A complete re-investigation of the development of the *T* / *T* embryo was outside the scope of the present study, which was primarily concerned with the V.E.R. However, some observations on the notochord of *T* / *T* embryos may here be put on record. The sectioned material available consisted mainly of 12 litters from *T* / + × *T* / + matings with nominal ages ranging between 8½–9½ days; these include 46 normal (+ / + and *T* / +) embryos, 14 *T* / *T* embryos, and 2 unclassifiable specimens. In addition, a few somewhat older *T* / *T* embryos from the tailless stocks (see next section) were also available.

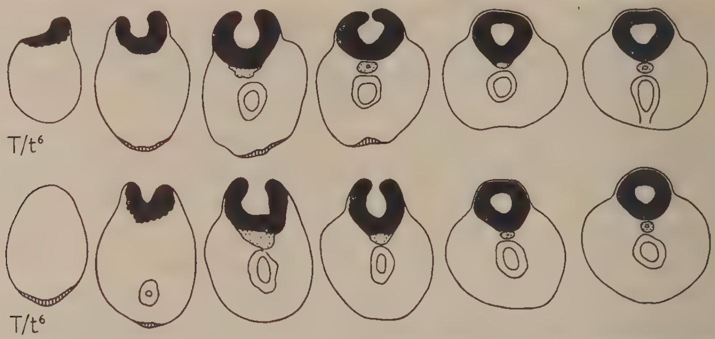
T / *T* embryos differ greatly from each other as regards the notochord. In some specimens, both before and after turning, no clear evidence for the existence of a notochord has been discovered. In others, groups of cells which lie either in the continuity of the roof of the gut or just dorsal to it suggest that they are parts of the notochord; taken by themselves, they could scarcely be regarded as critical evidence for the existence of a notochord in T/T embryos. However, in a minority of such embryos, the existence of a notochord as an identifiable separate entity is beyond doubt (though, of course, it is very far from normal). For instance, in one $8\frac{1}{2}$-day embryo which is clearly identified as T/T by paired dorsal blebs, the notochord is traceable first as a ribbon of thicker cells in the middle of the roof of the gut; then, for a number of sections, it occupies a position between gut and neural plate, and ultimately it is lost in the latter. Similar evidence from a fully turned 9-day embryo is illustrated in Plate 3, figs. 26–31. This is the least abnormal turned T/T embryo encountered in the present series; its diagnosis rests on the presence of pyknotic granules in the rear part of the embryo and a large and abnormally shaped neural tube with anomalies all along its length, neither of which ever occur in $T/+$ (or $+/+$) embryos. In this specimen, notochordal material projects distally from the ventral surface of the neural plate and tube (figs. 26 and 27); unlike $T/+$ embryos, this mass of cells shows advanced pyknosis; more anteriorly, the notochord is separate from the neural tube but still full of pyknotic granules (fig. 28); cranial to this (figs. 29 and 30) the notochord is for a short stretch a clearly identifiable and separate structure, but soon (fig. 31) it is incorporated into the ventral wall of the neural tube where it soon becomes unrecognizable.

To sum up. Whereas in the majority of T/T embryos there is either no identifiable notochord at all, or only somewhat problematical groups of cells, there are some individuals in which there is clear-cut evidence for the existence of a notochord as an identifiable separate structure; it is closely associated with the roof of the gut, or with the ventral side of the neural plate or tube, or with both; in addition, pyknotic processes may occur in it.

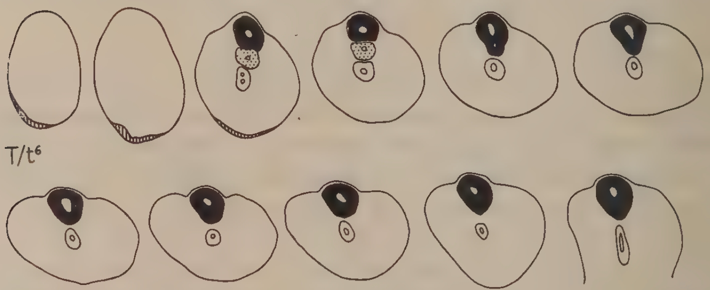
ANURY (T/t^6 and T/t^1)

The tailless genotypes whose development has been investigated by Gluecksohn-Schoenheimer (1938) are T/t^0 and T/t^1. In the present study, T/t^6 has been used in place of T/t^0. The t^6 'allel' was discovered by Carter & Phillips (1950). Soon afterwards Dunn & Gluecksohn-Schoenheimer (1950) reported that 'No combinations t^0/t^6 have yet been found (from crosses of $T/t^0 \times T/t^6$) and so t^6 is not distinguishable from t^0 and may be a recurrence of t^0.' T/t^6 mice were obtained from Dr. T. C. Carter and T/t^1 mice from Professor L. C. Dunn. The following report is mainly based on two litters from $T/t^6 \times T/t^6$ matings which include 6 T/t^6 and 4 T/T embryos; certain ancillary data, including 11 T/t^6 embryos, will not be considered separately, as they are in complete agreement with the rest. In addition there is one litter from a $T/t^1 \times T/t^1$ mating which contains 5 T/t^1 and 1 T/T embryos. This rather small material is adequate as the situation encountered is simple, and as a single point only had to be cleared up. As the two types of taillessness agree with each other, it will be sufficient to describe one of them.

The development of T/t^6 differs from that of $T/+$ embryos in degree rather than in kind. As shown in Text-fig. 5, the notochord is of increased bulk not only in its distal growth zone, but in the whole tail, in the cloacal region and even somewhat more anteriorly in the lumbar region. Lumina are usually large and tend to be traceable well into the cloacal region. The thickness of the distal parts of the notochord suggests that, as often in $T/+$ embryos, it may incorporate extraneous materials. However, unlike $T/+$ where such materials are usually soon hived off and re-incorporated into the neural tube (Text-figs. 1 and 2), the caudal notochord of T/t^6 remains intact and is incorporated as a whole into the neural tube.[1] The beginning of this process is shown in the neighbourhood of section 50 in both embryos of Text-fig. 5; often, this process starts in several places simultaneously. Soon, the notochord of the tail region disappears more or less completely into the neural tube; in the $10\frac{1}{2}$-day embryo of Text-fig. 6 the notochord



TEXT-FIG. 5. Sections 10, 20, . . ., 60 through the tails of two T/t^6 embryos (litter mates, nominal age $9\frac{3}{4}$ days; C.R.L. 3·6 and 3·7 mm. respectively).



TEXT-FIG. 6. Sections 10, 20, . . ., 110 through the tail of a T/t^6 embryo (nominal age $10\frac{1}{2}$ days; C.R.L. 4·5 mm.).

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[1] In at least one T/t^1 embryo the massive notochord becomes separated into a dorsal and a ventral mass in the region of the cloaca, with re-incorporation of the dorsal moiety into the neural tube.

of the tail is a separate structure near the tip of the tail only. Cranial to the tail the notochord is either a separate structure throughout, or it may be in contact with the cloaca or actually be retained in the roof of the cloaca for some distance, again as in $T/+$ embryos. The ventral ectodermal ridge of the tail of T/t^6 and T/t^1 embryos is reduced in size as in $T/+$ embryos, though possibly a little more so (Table 3 above). Regressive changes happen earlier in tailless than in $T/+$ embryos; pyknotic granules are present in the tails of T/t^6 and T/t^1 embryos as early as $9\frac{3}{4}$–$10\frac{1}{2}$ days, whereas in $T/+$ they do not appear until later.

DISCUSSION

The facts described in the preceding sections leave no doubt that in the $T/+$ embryo a notochord is present throughout the length of the body as in a normal embryo. Its absence from certain sections is apparent rather than real. It may be retained in the roof of the gut or cloaca for varying distances and usually for a limited period only. It may be slow to separate from the overlying neural plate or neural tube. Or it may secondarily become incorporated very completely either into the neural tube or into the tail-gut. However, if the notochord enters a structure, no matter how complete its histological assimilation, it generally re-emerges from the same structures somewhere else, and a study of serial sections leaves no doubt as to its essential continuity. The same situation obtains in tailless (T/t^6 and T/t^1) embryos. A notochord is present throughout the length of the tail as in a normal embryo; soon, however, it becomes incorporated into the neural tube throughout the tail and often the cloacal region.

The same, in principle, seems to be happening in the T/T embryo. There is no doubt that some, at least, of these embryos have an identifiable notochord. It seems, however, that in the majority of cases it is either more or less completely retained in the roof of the gut; or that it leaves the gut only to merge into the neural plate or neural tube; and, finally, that it tends to disintegrate by pyknosis. The behaviour of the notochord of the T/T embryo can thus be understood as a more extreme version of that in $T/+$ embryos: on this interpretation, T/T embryos do not lack the notochord, but the notochord is usually retained more or less completely in the roof of the gut or incorporated in the neural tube.

If the notochord tended to stick to, or become incorporated in, the neural tube only, there would be no reason to blame one rather than the other for this tendency. The fact that the abnormalities of the notochord may involve either of its neighbours (or both) suggests that the notochord is the structure primarily at fault. This does not necessarily mean that the neural and digestive systems are quite normal. A study of the sectioned material of $T/+$ embryos suggests that the common denominator for all the abnormalities of the notochord may be a change in its surface properties. Supposing the notochord of $T/+$ embryos were abnormally 'sticky', this might explain both its tendency to be retained in the gut, &c., and to become attached secondarily to its neighbours; it might also explain the apparent tendency of the $T/+$ (and T/t) notochord, on differentiating out of the primitive streak, to take with it materials normally destined to become neural tissue. A change in the consistency and surface structure of the notochord might also explain the tendency to form a lumen[1] and perhaps also the tendency to form branches which sometimes re-coalesce with the main stem of the notochord.

The present data on $T/+$ and T/t embryos seem to suggest that the notochord is the structure primarily affected by the T gene. The neighbouring structures such as neural tube and gut do not show any abnormalities except where these are explicable by the behaviour of the notochord. The ultimate regression of the tail tip in $T/+$ and of the whole tail in T/t embryos is clearly a consequence of the abnormalities of the notochord in these parts of the axial skeleton. The situation is less clear in the T/T embryo. The present author would be rather more hesitant to blame all the massive abnormalities of the posterior trunk region on the notochord alone. These major defects would seem to implicate the primitive streak as a whole. Perhaps the notochord is involved as part of a more general disturbance of the primitive streak which does not become obvious as such except in the T/T homozygotes. A disturbance of the primitive streak as the possible cause behind more obvious anomalies of the notochord has recently been suggested in the case of Danforth's short-tail ($Sd/+$ and Sd/Sd) in the mouse (Grüneberg, 1958).

Unlike the rather trivial involvement of the V.E.R. in $Sd/+$ and Sd/Sd embryos, its reduction in $T/+$ and T/t embryos cannot be ignored. T/T embryos do not form the tail region on which, in normal development, the V.E.R. is situated; they are, however, greatly abnormal long before the time at which the normal embryo forms the V.E.R. Similarly, abnormalities of the notochord are present in $T/+$ embryos before a V.E.R. is present. It is thus clear that the reduction of the V.E.R. cannot be responsible for the abnormalities of the notochord. Short of assuming that the reduction of the V.E.R. is a second primary gene effect and thus constitutes a case of genuine pleiotropism (for which there is clearly no evidence), it must be concluded that the involvement of the V.E.R. is secondary. Why the other tail abnormalities lead to a reduction in size of the V.E.R. is so far obscure. Perhaps, as has been postulated for the apical ectodermal ridge of the limb-buds in the chick (Zwilling & Hansborough, 1956), there is a reciprocal relationship such that a mesodermal factor (disturbed in $T/+$, &c.) is responsible for maintaining the V.E.R. in an active state.

Where, as in the present case, embryological studies are separated from each other by a span of 20 years or more, differences in the genetic background may be responsible for some of the divergencies encountered. The *T* / + mice of the present series were, if anything, rather more mildly affected than those investigated by Chesley (1935). His careful descriptions of the *T* / *T* embryo are in complete agreement with the present observations. In particular, Chesley made it quite clear that in early stages, many *T* / *T* embryos

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[1] Though no lumina of the notochord have ever been seen by me in mouse embryos not carrying the T gene, the presence of a lumen cannot in itself be regarded as pathological. The presence of a lumen in the notochord of presumably normal human embryos has recently been described by Sensenig (1957).

> show material in the posterior half which is probably notochordal. This material often lies in the dorsal wall of the gut as does the notochord in the normal embryo. . . . In none of these embryos is the material as regular as is the notochord in normal embryos at this age, however. Thus in embryo no. 40–8 (8 days 17 hours) . . . this tissue is larger than normal posteriorly, and lacking anteriorly. The section figured shows a group of cells which, if regular in the region anterior and posterior to the section, would certainly be interpreted as notochord. This is not the case, however. The portion shown in the figure definitely is *continuous anteriorly with the neural tube.* It arises in a normal fashion from the primitive pit posteriorly. [p. 436; italics mine.]

Chesley thus observed the merging of the notochord with a neighbouring structure, and similar cases are described elsewhere.

> It is clearly indicated from this series of homozygotes that the notochord-forming region is at first morphologically normal, but that the differentiation of chorda dorsalis is abnormal although not entirely lacking in the early stages. By the day during which death occurs no trace of notochord is present. [p. 437.]

The present observations are in fair agreement with Chesley's description of the *T* / + embryo, so far as it goes. Discontinuities of the notochord are mentioned particularly in an $8\frac{3}{4}$-day-old embryo where 'the notochord is very definite and normal in the anterior and posterior regions, but cannot be found in the mid-region. The neural tube is markedly abnormal in its ventral part and could thus conceivably obliterate the notochord' (p. 443). Another instance is a *T* / + embryo of 10 days 14 hours, where 'the notochord cannot be detected for a considerable distance in the posterior limb bud and proximal tail region' (p. 445). At a later stage, 'The filament at the distal end of the tail of heterozygous embryos shows somites, but is lacking in neural tube and notochord' (p. 453). Chesley nowhere suggests that the level at which the constriction is formed is determined by the end of the notochord (or, indeed, by that of the neural tube) and hence that the distal part of the tail which becomes the filament did not contain a notochord (and neural tube) in the first instance; he thus does not discuss the question of whether the absence of these structures from the filament is primary or secondary, i.e. due to regression following the formation of the constriction; though he describes several pre-filament embryos with a notochord of normal length. Our own finding that the notochord of *T* / + embryos is at first as long as in normal embryos is thus not in disagreement with Chesley's original story.

Curiously enough, the presence of a lumen in the *T* / + notochord is not mentioned by Chesley. It can scarcely be assumed that so careful an observer should have overlooked such an obvious feature. However, as the lumen occurs in our $T/+$ stock and in both tailless stocks which are of quite different origin, it is hard to believe that the lumen should have been completely missing from the $T/+$ stock maintained in Columbia University in the early 1930's. Perhaps the lumen has been overlooked because it was smaller in size and less regularly present.

Chesley recognized that the notochord was probably the basic structure affected by the *T* gene; he also considered the possibility that the primitive streak might be ultimately to blame. However, he did not penetrate to an understanding of the pathological mechanism involved and hence had difficulties in seeing the common features in the development of $T/+$ and T/T embryos. The present investigation thus carries Chesley's work a step further.

The development of taillessness (T/t^0 and T/t^1) has been investigated by Gluecksohn-Schoenheimer (1938). That author described connexions between notochord and hind-gut and/or cloaca, and rightly suggested that these might be due to a 'failure of the notochord to separate entirely from the gut' (p. 580). The behaviour of the notochord in the tail region is described as follows (p. 579):

> There exists thus a striking difference between the tail bud and the elongated tail of the normal and of the mutant mouse. While in the normal mouse all structures present in the tail bud are drawn out into the elongated tail, the elongated tail of the future tailless mouse does not contain all of the structures present in the tail-bud; it lacks the notochord. It seems that the notochord has reached its furthest posterior extension in the tail *bud* of the mutant embryo; it does not lengthen during further growth of the tail. [Italics in the original.]

Again, on p. 583, the author emphasizes that 'the elongated tail of the tailless mouse *never* contains a notochord' (italics in the original). This description is thus at variance with our own findings. In our material, the notochord at first extends fully into the elongated tail of future tailless embryos; it later disappears from sight by hiding in the neural tube. In discussing the pathological processes of $T/+$ and tailless embryos, the author further states that 'The same kind of malformation of the notochord is observed in both of them, shortening of the notochord . . .' (p. 581). As pointed out above, Chesley never discussed the question of whether the absence of notochord and neural tube from the tail filament of $T/+$ embryos was primary or secondary. His work thus cannot legitimately be quoted in favour of a primary absence of the notochord from the tail filament in $T/+$. The present study clearly shows that the notochord is just as long, at first, as in a normal mouse. The same, in our material, applies to tailless embryos. What $T/+$ and T/t embryos have in common is the fact that the caudal notochord becomes incorporated in a neighbouring structure to a greater or lesser extent.

The question arises as to whether the discrepancy between the findings of Gluecksohn-Schoenheimer (1938) and the present paper can be explained by a difference in genetic background. The T/t^1 stock obtained from Professor L. C. Dunn and the T/t^6 stock obtained from Dr. T. C. Carter agreed closely with each other although they are of completely different origin. Moreover, both of them agreed in the critical feature with the $T/+$ stock. Under these circumstances, a difference in the genetic background does not appear to be a plausible explanation.

In 1942 Dunn established the remarkable fact that when $T/+$ mice are repeatedly back-crossed to the inbred Bagg albino strain, the manifestation of the *T* gene is reduced, and the tails of the $T/+$ mice become more nearly normal. If, on the other hand, $Sd/+$ mice are repeatedly back-crossed to the same inbred strain, the manifestation of the *Sd* gene is enhanced, and most of the $Sd/+$ heterozygotes become tailless. From this he concluded that 'when two mutations operate through different channels, their effects are liable to modification by different factors, and each will thus appear to have dominance modifiers specific to itself'. Facts which have since become available suggest the possibility of an alternative hypothesis, namely, that although two genes act through different channels, they may yet be modified in different directions by the same 'modifying genes'. As shown previously (Grüneberg, 1958), the growing end of the notochord in $Sd/+$ embryos is reduced in size; as shown in this paper, the growing end of the notochord in $T/+$ embryos is increased in size. Supposing the Bagg albino strain contains a gene (or genes) tending to make the distal end of the notochord smaller: then that gene would tend to enhance the effect of *Sd*, but at the same time it would tend to diminish the effect of *T*. It need scarcely be pointed out that, while this is a definite possibility, and may even be plausible, there is no evidence that this is what actually happened when Dunn (1942) transferred the *Sd* and *T* genes on to the genetical background of the Bagg albino strain.

It appears that *T* and *Sd* are genes with a somewhat similar mode of action. In both cases the notochord is deeply involved though, again in both cases, there is some evidence that the ultimate fault may lie with the primitive streak. In *T* there is a prima facie case for the assumption of a change in some surface property of the notochord; contacts with neighbours are abnormally long maintained or established afresh. The same applies to *Sd*, where separation from the paraxial mesoderm is delayed. It would be of great interest if the surface properties of the notochords of these mutants could be directly compared with those of the normal notochord in tissue culture or by some similar technique.

SUMMARY

1. The notochord of $T/+$ mouse embryos is of normal length. In young embryos it tends to be retained to a varying extent in the dorsal wall of the hind-gut and/or the cloaca. Often, its separation from the primitive streak and/or the overlying neural plate is also delayed. In older embryos the notochord tends to become incorporated either in the neural tube or in the tail-gut

in the distal region of the tail. The notochord of $T/+$ embryos often has a central lumen and tends to branch.

2. In the majority of T/T embryos there is either no evidence, or ambiguous evidence, for the existence of a notochord as a separate structure. Some T/T embryos, however, show clear evidence of a notochord though this tends to be retained in the gut or to become incorporated in the neural tube.

3. In T/t^6 and T/t^1 tailless embryos the caudal notochord is of normal length; however, it soon becomes incorporated in the neural tube.

4. It is suggested that the action of the T gene in all these genotypes may be a change in some of the surface properties of the notochord.

5. The possibility is considered that the notochord may be involved as part of a more general disturbance of the primitive streak.

ACKNOWLEDGEMENTS

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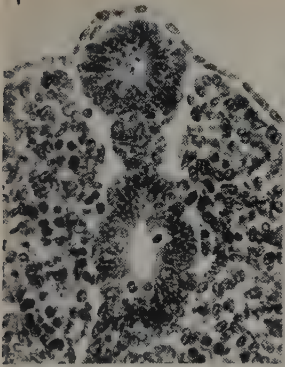

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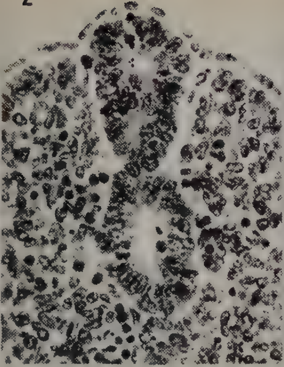

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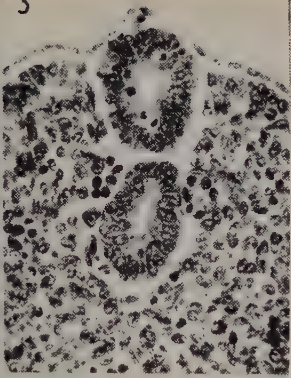

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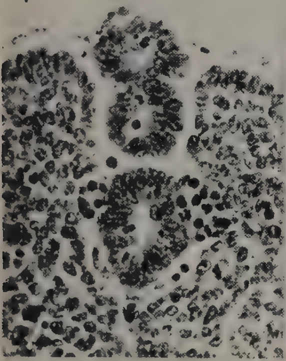

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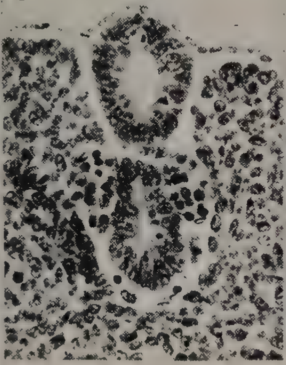

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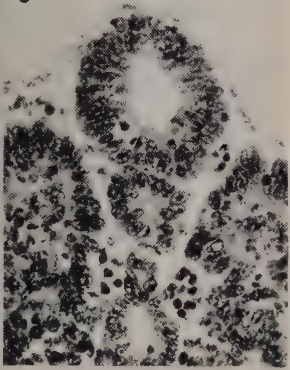

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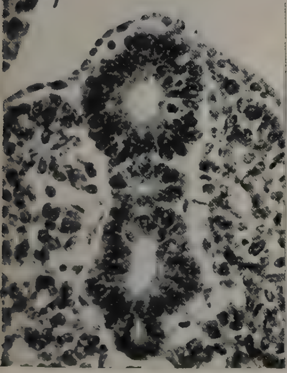

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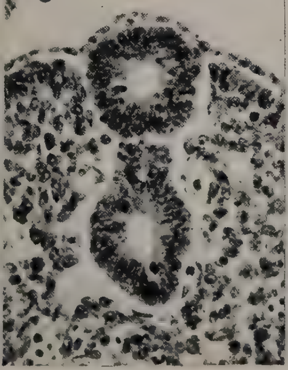

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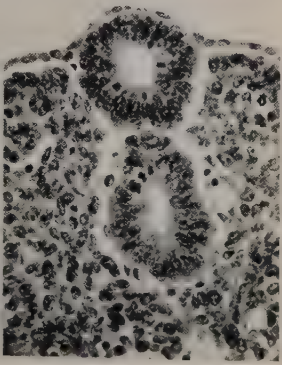

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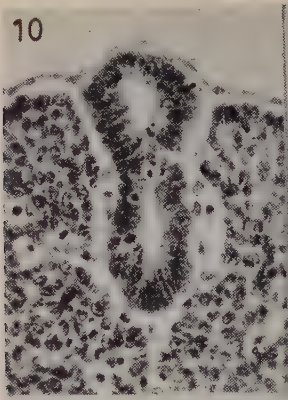
Plate 1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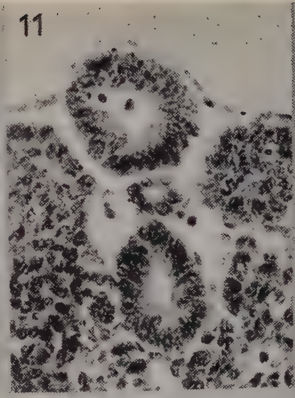
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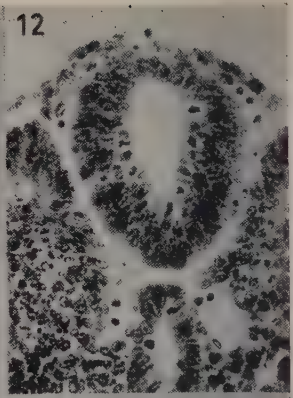
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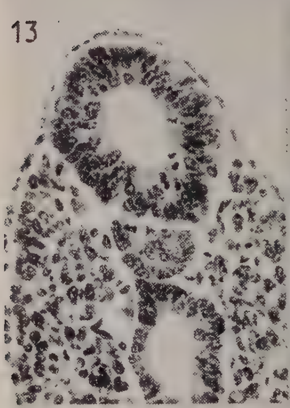
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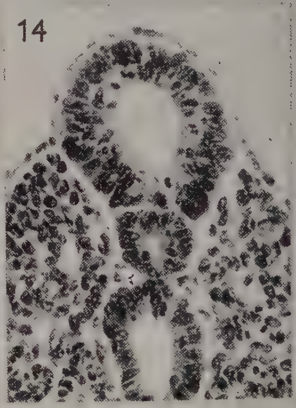
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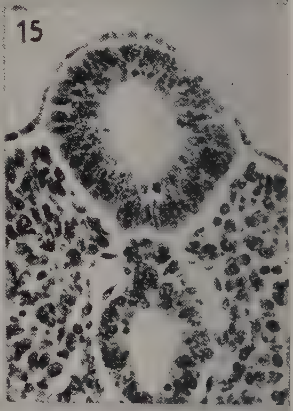
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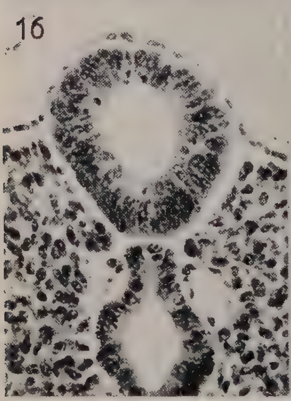
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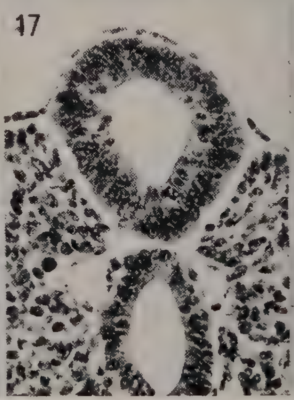
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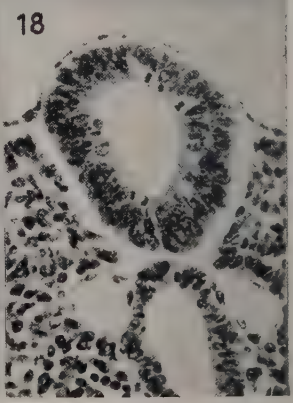
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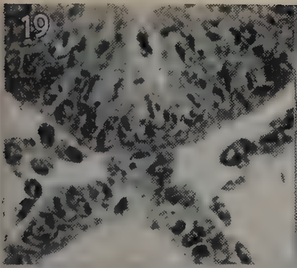
Plate 2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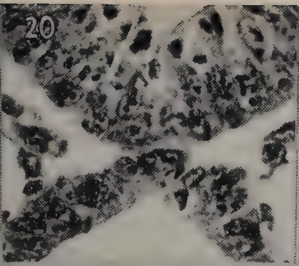
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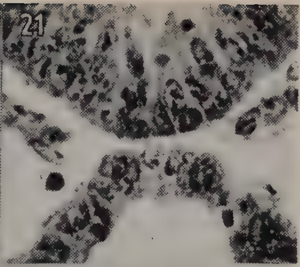
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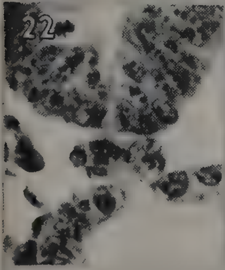
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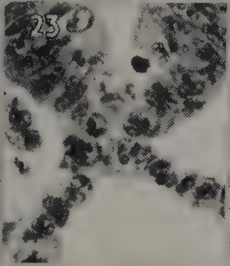
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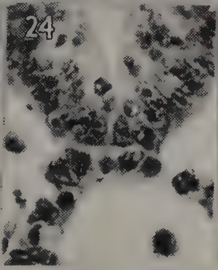
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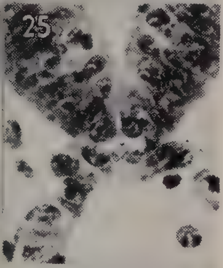
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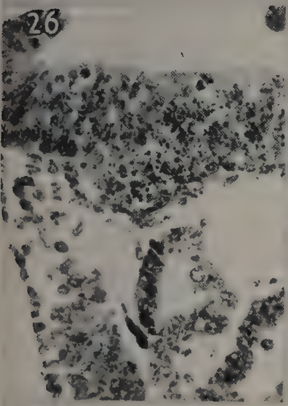
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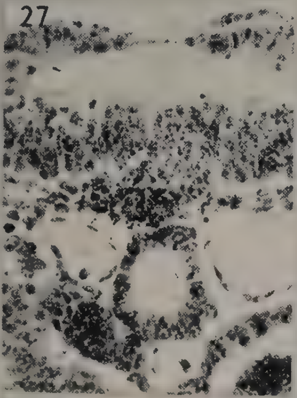
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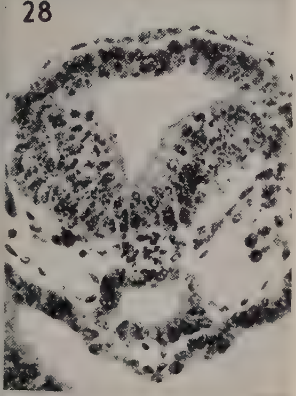
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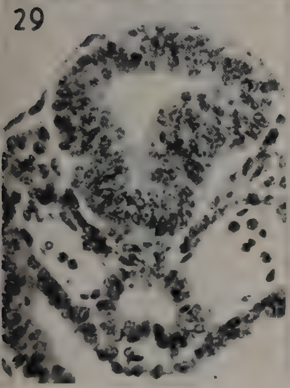
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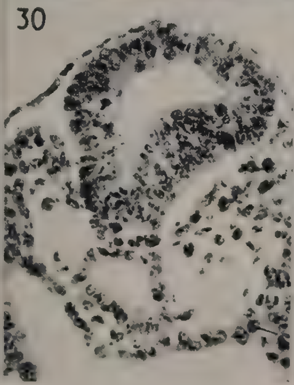
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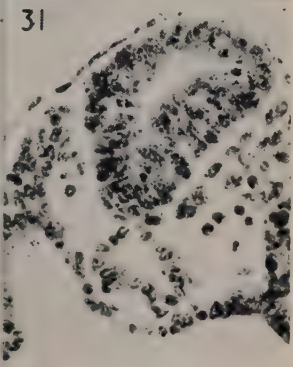
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Plate 3

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EXPLANATION OF PLATES

All microphotographs are transverse sections through the tail or the posterior trunk region of mouse embryos. Bouin fixation; embedded by Peterfi's method. The sections in figs. 7–12 are 10 μ thick; all others are 7·5 μ. Haematoxylin and eosin. Magnification of figs. 1–18 and 26–31: ×250; of figs. 19–25: ×450.

PLATE 1

FIGS. 1–6. $T/+$ embryo (litter 16; nominal age 10½ days; C.R.L. 4·4 mm.; sections 30, 33, 44, 52, 62, and 99 as counted from the tail tip).

FIGS. 7–9. $T/+$ embryo (litter 16; nominal age 10½ days; C.R.L. 4·3 mm.; sections 24, 29, and 33; continued on Plate 2).

PLATE 2

FIGS. 10–12. $T/+$ embryo (continued from Plate 1, figs. 7–9; sections 38, 58, and 95).

FIGS. 13–18. $T/+$ embryo (litter 20; nominal age 10 days; C.R.L. 3·5 mm.; sections 32, 39, 55, 59, 64, and 75).

PLATE 3

FIGS. 19–21. $T/+$ embryo (litter 23; nominal age 9½ days; C.R.L. 2·8 mm.; sections 60, 66, and 72).

FIGS. 22–25. $T/+$ embryo (litter 28; nominal age 9 days; C.R.L. 1·7 mm.; sections 71, 73, 75, and 77).

FIGS. 26–31. T/T embryo (litter 33; nominal age 9 days; mean C.R.L. of five normal litter mates 2·26 mm.; sections 26, 31, 43, 49, 57, and 68).

(Manuscript received 5:ii:58)

Influence de la méthode d'élevage sur la différenciation nerveuse d'un fragment d'ectoblaste

par H. DENIS[1]

Laboratoire de Morphologie des Chordés, Université de Liège

AU cours d'un récent travail (Denis, 1957), exécuté sur *Pleurodeles waltii*, nous avons soumis un fragment d'ectoblaste prélevé au pôle animal d'une jeune gastrula, à l'influence inductrice du chordo-mésoblaste troncal d'une jeune neurula (stade H. 14–15). Ce greffon, quand il est implanté sur le ventre d'un hôte du même stade, peut se neuraliser entièrement. Les protocoles d'expériences amenaient à conclure qu'un contact inducteur de durée très réduite doit suffire pour amorcer la transformation nerveuse de l'ectoblaste. La neuralisation, cependant, est loin de se produire dans toutes les expériences: elle exige, pour se réaliser, des conditions bien spéciales: il faut que le greffon demeure ramassé sur lui-même et résiste à l'étirement provoqué par l'allongement de son hôte.

En vue de vérifier les conclusions du travail précédent, une nouvelle série d'expériences a été entreprise. Le problème était de fournir au greffon des conditions d'élevage favorables à sa neuralisation. En l'occurrence, il s'agissait d'empêcher le greffon de s'étirer sur le ventre de son hôte.

La technique d'implantation dans le blastocoele mise au point par Mangold (1929) permet de placer un greffon dans la région hépatique, non en surface mais en profondeur. Mangold a démontré que dans ce cas, un fragment d'ectoblaste n'ayant pas subi de contact inducteur s'incorpore aux tissus de l'hôte. D'autre part, le fragment placé dans le blastocoele, en avant de la future ébauche hépatique, ne subit aucun étirement car ce phénomène est surtout actif dans les tissus superficiels de l'hôte.

MÉTHODE

Un fragment d'ectoblaste prélevé au pôle animal d'une jeune gastrula de *P. waltii* a été implanté dans la plaque neurale d'une jeune neurula de la même espèce (stade H.14–15). L'implantation fut faite dans la région troncale antérieure. Le greffon est demeuré en contact avec la chorde et les somites de son hôte pendant une durée de 3 heures (élevage à 12° C.). Après ce laps de temps, il est repris, soigneusement débarrassé de toute cellule mésodermique adhérente et

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introduit dans le blastocoele d'une jeune gastrula de même espèce. Pour cela, une incision est pratiquée au pôle animal de l'hôte et le greffon est poussé par cet orifice du côté opposé à l'encoche blastoporale.

RÉSULTATS

Après une dizaine de jours d'élevage, les embryons opérés ont atteint le stade H.40–42 et, en général, présentent sur le ventre, dans la région troncale antérieure, un renflement qui marque l'emplacement du greffon.

L'étude microscopique a porté sur une vingtaine d'embryons. Les résultats sont consignés en un tableau.

TABLEAU

Nombre d'expériences	*Tissus obtenus*					
	Mésenchyme	*Mélanophores*	*Ganglions*	*Tissu neuroïde*	*Tissu nerveux*	*Organes des sens*
20	17 85%	18 90%	2 10%	2 10%	13 65%	1 (?) . .

Dans 7 cas sur les 20 étudiés, l'embryon porte-greffe présente, entre la peau et l'intestin antérieur, un gros amas nerveux, le plus souvent traversé de substance blanche et entouré de mélanophores. En un seul de ces cas, le massif nerveux se trouve au contact d'une sorte d'ampoule offrant une certaine ressemblance avec une capsule auditive embryonnaire. Dans tous les autres, les seules formations annexes au nodule nerveux sont des dérivés de crête neurale: mélanophores et cellules ganglionnaires.

Dans 6 autres cas, le greffon a donné une formation nerveuse de volume assez faible. Quant aux 7 embryons restants, ou bien ils ne portent aucune trace du greffon, ou bien ils présentent entre l'épiderme et l'intestin une certaine accumulation de cellules pigmentaires. Dans 2 cas seulement, l'hôte est parfaitement normal et tout porte à croire que le greffon a été entièrement assimilé et s'est intégré au mésenchyme voisin.

La série expérimentale actuelle montre donc que, environ 2 fois sur 3, un fragment d'ectoblaste compétent mis, durant 3 heures à 12° C., au contact du chordo-mésoblaste d'une neurula et transplanté ensuite dans le blastocoele d'une jeune gastrula, se transforme, en tout ou en partie, en tissu nerveux.

DISCUSSION

Ce résultat confirme la conclusion de notre travail précédent: un contact inducteur de courte durée (3 heures à 12° C.) suffit pour amorcer la transformation nerveuse de l'ectoblaste. Cette conclusion est en accord avec celle de Johnen (1956) qui trouve qu'un contact inducteur de 4 heures chez *Triturus vulgaris* est suffisant pour provoquer dans l'ectoblaste la formation de structures cérébrales.

D'autre part, dans 90 pour cent des cas, le greffon a fourni une grosse quantité de cellules pigmentaires, ce qui pourrait être interprété comme une confirmation de l'idée que les dérivés de la crête neurale constituent un stade intermédiaire dans la transformation de l'ectoblaste en tissu nerveux (Damas, 1947).

Par ailleurs, il est intéressant de comparer les résultats obtenus suivant les méthodes d'élevage utilisées. Dans le cas d'une implantation en surface, sur le ventre d'une jeune neurula, après un contact inducteur de durée identique, le pourcentage de neuralisation fut d'environ 11 pour cent (Denis, 1957). Après introduction du greffon dans le blastocoele, du tissu nerveux a été obtenu dans 65 pour cent des cas. Cette proportion plus grande ne peut être attribuée à l'action inductrice de l'ambiance entourant le greffon, car, comme Mangold (1929) l'a démontré, un fragment d'ectoblaste banal placé dans le blastocoele d'une jeune gastrula ne se transforme jamais en tissu nerveux. Elle doit donc plutôt indiquer que, introduite dans le blastocoele, la greffe n'a pas été empêchée de répondre à l'induction reçue. La différence essentielle entre les deux types d'expériences (greffe en surface et introduction dans le blastocoele) nous paraît être que, dans le dernier cas, l'implant demeure ramassé sur lui-même. En surface, il est étiré par l'épiderme de l'hôte qui s'étale sur une larve en allongement rapide.

Les expériences rapportées ci-dessus montrent une fois de plus, qu'une simple modification des techniques d'élevage peut transformer les résultats dans des proportions considérables. Aucune technique d'élevage ne peut donc être qualifiée de 'neutre'. Les résultats contradictoires obtenus par les différents auteurs s'expliquent bien souvent par l'utilisation de procédés qui ne sont pas comparables.

RÉSUMÉ

Soumis à l'influence inductrice du chordo-mésoderme troncal durant 3 heures à 12° C., un fragment d'ectoblaste compétent de *P. waltii* implanté ensuite dans le blastocoele d'une jeune gastrula de même espèce s'est transformé dans 65 pour cent des cas en tissu nerveux. Lorsque le même fragment est greffé sur la paroi ventrale d'une jeune neurula de même espèce, il ne donne du tissu nerveux que dans 11 pour cent des cas. Les conditions d'élevage peuvent donc favoriser ou inhiber la réponse d'un implant à l'induction.

SUMMARY

A fragment of competent ectoblast of *P. waltii*, subjected to the inductive influence of trunk chorda-mesoderm for 3 hours at 12° C. and then implanted in the blastocoel of a young gastrula of the same species, is transformed to nervous tissue in 65 per cent. of cases. When the same kind of fragment is grafted into the ventral surface of a young gastrula of the same species, it only formed nervous tissue in 11 per cent. of cases (Denis, 1957). The conditions in which it develops can, therefore, promote or inhibit the response of an implant to induction.

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The Forces Producing Neural Closure in Amphibia

by G. G. SELMAN[1]
From the Institute of Animal Genetics, Edinburgh

WITH ONE PLATE

IT has been shown by Smith (1946), working with trout eggs, that when an embryo develops the heat lost to the surroundings is balanced by the loss in the calorific value of its energy-rich storage substances. Other workers (e.g. Shearer, 1922) have shown that the heat lost by eggs and developing embryos is approximately equal to the available energy deduced from the observed respiration. It would seem, therefore, that only a very small fraction of the energy known to be available to the embryo from its aerobic metabolism can be stored as potential energy in the visible pattern of tissue structures which the embryo progressively develops. It has sometimes been concluded from this that little energy can be required for morphogenesis. This conclusion is doubtless true, but the argument is unsound, because no knowledge of the energy balance between the total energy being released by the embryo and the energy being lost to the surroundings can tell us anything about the work which may be required to overcome a potential energy barrier. For instance, consider this example which is used here as a simple illustration of the argument rather than as a model of what actually happens. It is conceivable that when a newt embryo gastrulates, considerable work must be done in so deforming the cells against their own elasticity that the blastula tissue may pass through the narrow blastopore opening. To perform such work energy is required from the chemical energy source; it will be stored as potential energy by deformed cells as they pass through the blastopore; yet when the constriction is passed, the cells relax to their equilibrium shapes once more and the energy is dissipated as heat. In such a case a knowledge of the energy-balance could tell us nothing about the work done in gastrulation. On the other hand, if we knew about the forces required to deform the cells we should be able to estimate the work required for gastrulation, using this particular model for the process.

Tuft (1953) working with *Rhodnius* made an indirect estimate of the proportion of the total energy available which could conceivably be used by an embryo in performing morphogenetic movements. As pointed out by Waddington (1956), one weakness of this estimate is the necessity of assuming a constant energy requirement for maintenance. Tyler (1942) found that two dwarf echino-

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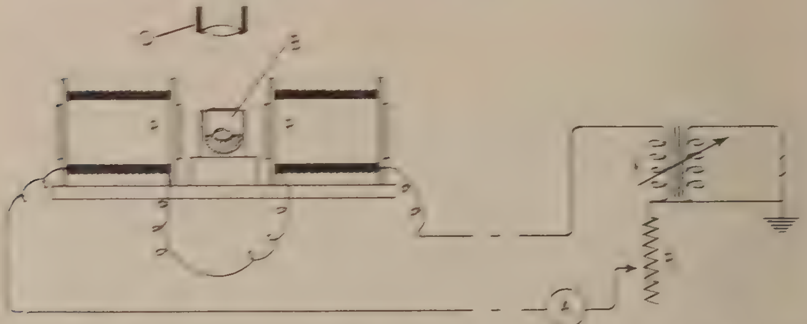
derm embryos respire at the same rate as one normal embryo of the same mass, but they take roughly 37 per cent. longer to gastrulate. Assuming maintenance requirements at a given stage are proportional to the living mass, this result may be interpreted as meaning either that two dwarfs require about 37 per cent. more energy for gastrulation than does one normal embryo (which agrees roughly with Tyler's theoretical estimate of 41 per cent.), in which case we can infer nothing about the relative magnitudes of the energies required for gastrulation and maintenance (see also Needham, 1942), or alternatively we may suppose that the energy needed for gastrulation is very small compared with that required for maintenance, in which case Tyler's results tell us nothing about the relative energies needed for gastrulation by dwarf and normal embryos. It seems likely that, to be in any sense reliable, an estimate must be based directly on measured values for the forces which the tissues of embryos can exert during their morphogenetic movements. The experiments of Moore (1941, 1945), who found the osmotically developed pressure within the blastocoel of an echinderm blastula which would just prevent gastrulation, led him to estimate that only a two-thousandth of the available energy from respiration would be required to perform the gastrulation process. The work required to change the shape of the embryo is evidently very small. Of course, considerable energy may be needed to produce the chemical syntheses associated with morphogenesis, but calculations based on physical measurements will give no information about this.

The present paper describes measurements of the forces which amphibian embryos can exert between their neural ridges during neural closure. The results lead to an estimate for the mechanical work done by an embryo during neurulation, and they provide data of value when one tries to consider how the neurulation process is carried out by the embryo. Such theories of neural closure as have been put forward in the literature have all attempted no more than a qualitative explanation of what is observed to take place. In future a satisfactory theory of neural closure should lead to a derivation of a neural closure force of the correct order of magnitude. The present paper is concerned, however, with the forces and work involved in neural closure rather than with theories of morphogenesis. A preliminary communication about this work was made by Selman (1955).

EXPERIMENTAL PROCEDURE

The method arose out of a suggestion made by Professor C. H. Waddington that it might be possible to apply the principle of the A.C./D.C. moving iron ammeter to the measurement of forces developed by embryos during morphogenesis. In the present experiment two small iron dumb-bells were mounted on the neural plate parallel to and against the neural folds. They were made to repel each other by arranging them in a uniform magnetic field produced by a pair of electromagnetic coils set up similarly to a Helmholtz arrangement (Text-fig. 1). The force of mutual repulsion between these induced magnets could be varied by adjusting the alternating current passing through the coils. By balancing this magnetically produced force against the force which the neurulating tissue could exert, an estimate of the latter could be obtained.

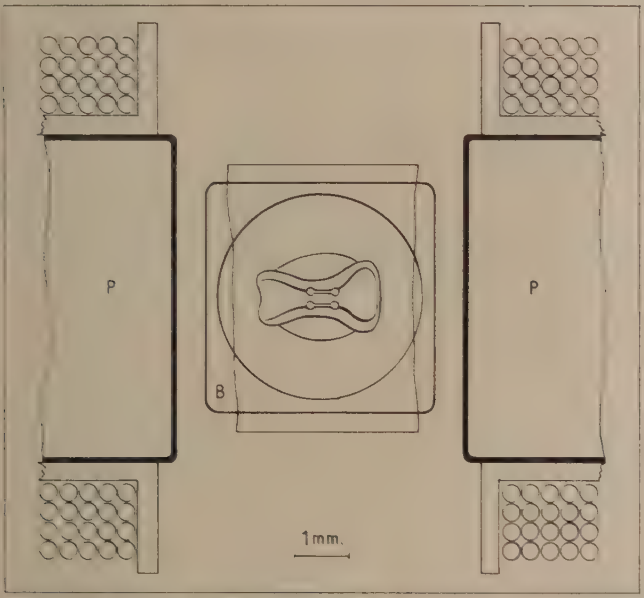
The dumb-bells themselves were found by careful searching in a single sample of iron shot which contained a majority of spherical iron particles. A typical dumb-bell might be described as two equal spheres or ellipsoids of diameter ~150 μ connected by a cylindrical shaft about 30 μ in diameter and 300 μ long



Text-fig. 1. The diagram shows in elevation the small perspex box *B* containing the neurula mounted beneath the microscope objective *O* and between the poles *P* of the two electromagnets. The circuit diagram shows the means by which the alternating current through the electromagnets is controlled by the rheostat *R* and auto-transformer *T* and measured with the milliammeter *A*.

(Plate, fig. A). The iron dumb-bells were approximately of similar form, but they varied in length *L* between about 450 and 800 μ, and were of mass *m* between 16 and 98 μg each. About 20 matching pairs of dumb-bells were selected on a basis of approximately equal weight, length, and general proportions.

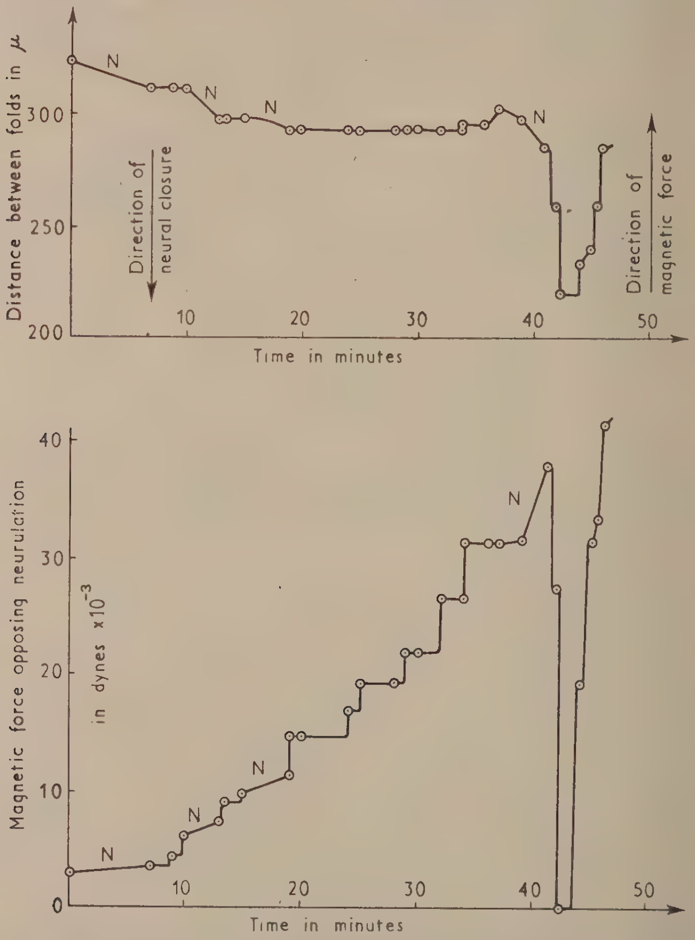
Embryos of two species were used, the newt, *Triturus alpestris*, and the axolotl, *Siredon mexicanum* (sometimes called *Amblystoma mexicanum*), because these possess well-developed neural folds before neural closure. After removal of the jelly capsule and vitelline membrane with fine forceps, the undamaged embryo was placed in 1/10th strength Holtfreter's saline, in a wax depression inside a small perspex box in the shape of a cube of side 5 mm. A pair of equal dumb-bells was then mounted symmetrically on the neural plate where the folds would normally first close. The box containing the neurula was then mounted between the two electromagnets so that the dumb-bells were approximately on the axis of the coils, midway between them, and parallel to the lines of force of an inducing magnetic field (Text-fig. 2). A small alternating current was then passed through the coils, so that the dumb-bells mutually repelled each other and pressed against the side of the neural folds (Plate, fig. C). After about 30 minutes the edges of the folds became partly moulded to the contours of the sides of the dumb-bells, and this helped greatly to reduce the tendency for the dumb-bells to slip sideways when greater magnetic fields were applied later. A fragment of coverslip was placed across the top of the plastic box and in contact with the saline surrounding the embryo, so as to provide a plane window for observation. Throughout the experiment the neural closure and the behaviour of the dumb-bells were followed, using a microscope giving a magnification of × 100 and having a scale in the ocular which enabled the separation D between the dumb-bells to be continually recorded. Knowing the separation D and the current i passing through the coils, the force of repulsion between the particular pair of induced magnets could be calculated by reference to graphs prepared in separate calibration experiments to be described later. Alternating current was used throughout all experiments in order to avoid difficulties due to residual magnetism which might otherwise have arisen. The value of the current was smoothly raised or lowered from zero using an auto-transformer and rheostat (Text-fig. 1). The coils had soft iron cores with plane parallel circular faces 6·8 mm. in diameter.



TEXT-FIG. 2. The small perspex box *B*, containing the neurula, is shown mounted between the pole pieces *P* of the electromagnets, so that the dumb-bells on the neural plate are on their axis and midway between them. The view shown is as seen through the observer's microscope.

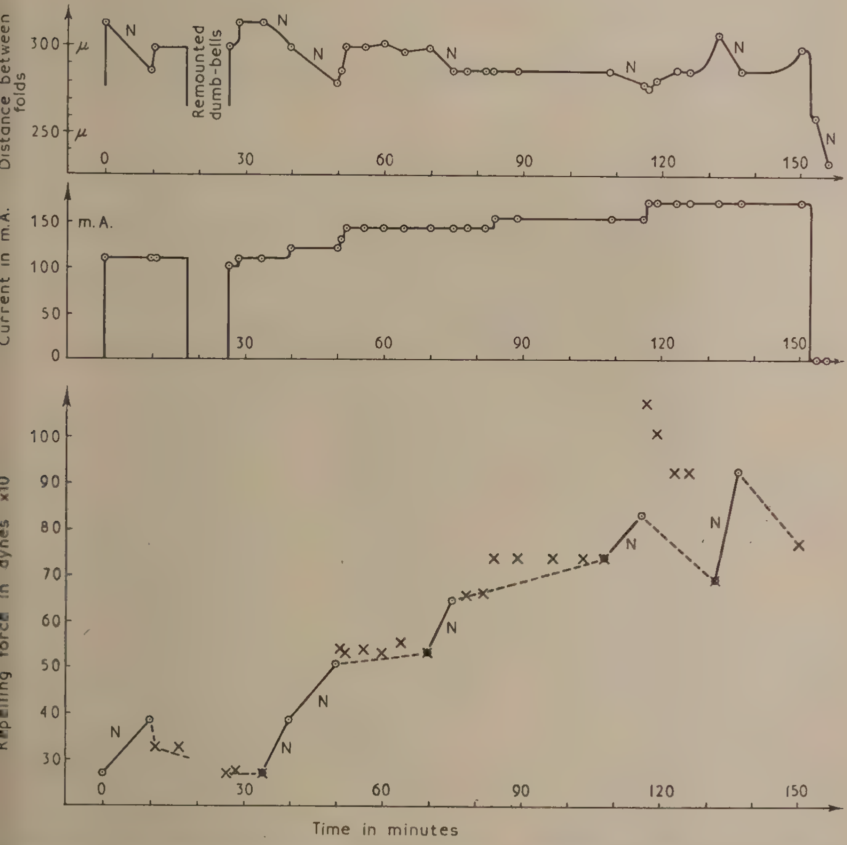
In the majority of experiments an attempt was made to hold the separation between the neural folds near the dumb-bells at approximately the same value by adjusting the current through the coils. Meanwhile, the neural folds would continue to close at distances greater than about 500 μ on either side of the dumb-bells, and in some experiments the neural closure took place in the head region while the mid-fold region was held apart by the force between the induced magnets.

If a movement towards neural closure took place, so that the dumb-bells were forced nearer each other, the neurulating tissue was then known to be exerting a force greater than, and opposite to, the force applied magnetically, whose magnitude was known from the values of current *i* and separation *D*. The neurulating tissue at this time was therefore capable of exerting a closure force at least as great as this. The movements towards neural closure which took place



TEXT-FIG. 3. The graphs refer to experiment 14 performed on a neurula of *Triturus alpestris*. The distances between folds plotted on the upper graph were measured values of $d+2r$, where *r* is the radius of the dumb-bell ends, while the magnetic forces opposing neurulation plotted on the lower graph were found from the observed values of *d* and current *i* by reference to the curves obtained with the same dumb-bell pair in the direct calibration experiment using the torsion-balance. Both curves shown here have the same time scale. *N* indicates a movement towards neural closure (see text).

uring experiment 14 with the newt, and experiment 13 with axolotl, are indiated against the letter *N* in Text-figs. 3 and 4 respectively. The corresponding umerical values, which have been calculated for the neural closure forces



EXT-FIG. 4. The graphs refer to experiment 13 performed on a neurula of axolotl. The upper and ower graphs are plotted like those in Text-fig. 3 for experiment 14, although the calibration mployed was indirect. The middle graph shows the current *i* passed through the coils of the lectromagnet during the experiment. In the lower graph the dotted line gives suggested values or the closure force which the neurula can exert. This force must be greater than, or equal to, the alues depicted by the points ⊙ recorded during neural closure, and less than, or equal to, the values shown by the points x when there was no closure (see text).

xerted by the embryo against the dumb-bell-shaped magnets, are shown in ables 1 and 2, also oppose the letter N.

It will be seen that the design of the experiment is such that when the current *i* s held constant, any neural closure movement meets increasing resistance as the umb-bells are carried nearer to each other by the neural folds. On the other hand, when the current was maintained at a constant value for a period of tim during which no movement towards neural closure took place, then the force o mutual repulsion between the magnets was known to equal or perhaps migh

TABLE 1

NEURULATION FORCES TRITURUS ALPESTRIS

No. of expt.	Force of neurulation in dynes × 10^{-3}	Time in minutes	Calib- ration	Separation of folds in μ	Notes
14	N ≥ 2·5 → 3·4 N ≥ 6 → 7 N ≥ 10 → 11 ≤ 15 → 27 N ≥ 31 → 38	0 — 7 10 — 13 15 — 19 19 — 34 38 — 41	DIRECT	324 → 311 311 → 298 298 → 293 293 298 → 285	Mid-fold
10	N ≥ 4 → 4·3 N ≥ 4·3 → 8·7 N ≥ 12 → 13 N ≥ 15 → 44 ≤ 46·5	0 — 7 7 — 23 24 — 27 35 — 44 48 — 56		381 → 368 368 → 276 263 → 255 255 → 219 237	Mid-fold
8	≤ 18 — 38 N ≥ 18 → 46	0 — 36 ~ 90		179 179 → 153	Mid-fold
2	N ≥ 39 → 40 ≤ 28 — 25	~ 30 40 — 90	INDIRECT	~ 205 ~ 210	Mid-fold
3	N ≥ 7·4 → 9 N ≥ 9 → 12·6 N ≥ 8 → 13 N ≥ 15 → 30 ≤ 50	3 — 8 8 — 28 41 — 43 48 — 74 74 — 78		313 → 294 294 → 268 294 → 263 250 → 227 229	Mid-fold
5	N ≥ 6 → 7·8 ≤ 7·3	55 — 60 60 — 70		427 — 375 427	Torn side Mid-fold
4	N ≥ 34 → 43	150-165		244 — 230	Mid-fold
9	≤ 21 — 40	10 — 50		161	Head region

be greater than the force which the neurula could exert. The calculated value for the magnetic repulsion forces under these experimental conditions are als listed in Tables 1 and 2; the forces which the neurula could then have exerted i closure were known to be less than or equal to these values.

When all these calculated values are plotted against time for a particula experiment (e.g. in experiment 13, see the lower graph of Text-fig. 4) they defin fairly precisely a curve, which shows how the force, which the neural fold exerted against the magnets in trying to produce neural closure, rose during mos of the time in which the experiment was made. This curve, of course, must pas through or below some points, and through or above others, depending upon the experimental conditions as described above.

It was found that the magnetically applied force necessary to keep the folds at approximately the same separation during at least the first 40 minutes of the experiments must be gradually increased with time. This is clearly illustrated

TABLE 2

NEURULATION FORCES AXOLOTL

No. of expt.	Force of neurulation in dynes × 10^{-3}	Time in minutes	Calibration	Separation of folds in μ	Notes
11	$\leqslant 10{\cdot}8$	0 — 60	DIRECT	481 — 586	Torn side Head region
	$N \geqslant 6 \longrightarrow 8$	135–145		350 — 402	
13	$N \geqslant 27 \longrightarrow 39$	0 — 10	INDIRECT	312 — 286	Mid-fold
	$N \geqslant 27 \longrightarrow 38$	34 – 40		312 — 299	
	$N \geqslant 38 \longrightarrow 52$	40 – 50		299 — 278	
	$\leqslant 52$ — 55	52 — 70		296 — 301	
	$N \geqslant 54 \longrightarrow 65$	70 — 75		299 — 285	
	$\leqslant 74$	75 — 109		285	
	$N \geqslant 74 \longrightarrow 83$	109 — 117		285 — 275	
	$\leqslant 92$	123 — 126		285	
	SUBSIDES TO 69	126 — 132		285 — 307	
	$N \geqslant 69 \longrightarrow 92$	132 – 137		307 — 285	
	SUBSIDES TO 77	137 — 150		285 — 300	
12	$N \geqslant 6 \longrightarrow 7{\cdot}5$	44 – 60		324 — 298	Torn side Mid region
	$\leqslant 7$ — 8	60 – 70		298 — 310	
7	$N \geqslant 48 \longrightarrow 65$	16 — 30		265 — 254	Head region
	$\leqslant 70$ — 87	30 – 47		251 — 265	
6	$N \geqslant 26 \longrightarrow 38$	0 — 32		445 — 405	Mid-fold
	$N \geqslant 76 \longrightarrow 106$	32 — 45		405 — 392	
	SUBSIDES TO 55	45 – 47		392 — 419	
	$N \geqslant 56 \longrightarrow 118$	47 — 74		419 — 314	

y experiment 14 (Text-fig. 3). This effect is partly a consequence of the elasticity f the neural tissues themselves and the manner in which most experiments were erformed. Under conditions where neural closure is opposed by an externally pplied force which is increased gradually, the neural closure effort is applied to ompressing the tissues which are trying to push the magnets together. In xperiments 2, 4, and 8, however, with neurulae of newt, much of the elastic ompression of the tissues was taken up at the beginning of the experiment by pplying a fairly large magnetic force, of the order 30×10^{-3} dynes, which naturlly opened the neural folds about 40 μ at the moment it was applied. Neverthe-ess, in these cases, at least 30 minutes had to elapse before a neural closure movement took place. Irrespective of how the experiment was performed, at least 30 minutes had to elapse before a closure force of the order 40×10^{-3} dynes could be exerted by the newt neurula.

For newt embryos, the maximum force tending to produce closure which the neural folds could exert against the dumb-bells was found to be between 40 and 45×10^{-3} dynes. The maximum force which the neural folds of an axolotl neurula could exert was found to be about 90×10^{-3} dynes in one case (experiment 13) and about 110×10^{-3} dynes for another case (experiment 6). It should be remembered that the embryo of the axolotl is larger than that of the newt.

Under the conditions of these experiments the neural closure movements, or increases in the force which the neural folds exerted against the magnets, were made intermittently between intervals of inactivity. Closure took place, or tended to take place, in steps each of about 13 μ. The maximum force which the neural folds could develop was likewise exerted intermittently between intervals when only a much smaller force could be produced. Then, while the current through the coils was held constant at a high value, the neural folds periodically opened a little as they moved apart under the magnetically applied force, then approached their former positions as the forces of neural closure increased to their former value, and then opened again. This behaviour is illustrated in experiment 13 (Text-fig. 4 and Table 2).

The measured values for the force exerted by the tissues during neural closure were independent of the lengths of the dumb-bell pairs used in the separate experiments. This result is an expression of the rigidity of the neural-fold tissue and provides confirmation that it is appropriate to express the neural-closure forces exerted by the tissues in dynes rather than in dynes per unit length of neural fold.

All experiments except two were carried out with the dumb-bell pair on the mid-fold region of the neural plate, where the folds are normally first to close. It was easiest to carry out the experiment in this position, because the dumb-bell shape fitted the contours of the embryo here, and the dumb-bells were therefore less liable to slip sideways. Two experiments, however, 7 and 9, were made on the head region of the neurula, but the results were insufficient to establish whether the neural folds in the head region could exert as great a closure force as in the mid-fold region.

The tissues of the neural folds showed elastic behaviour. For newt, a repulsion force 40×10^{-3} dynes opened the mid-folds about 50 μ. A plot of applied force against the resulting separation was roughly linear up to this point. The folds returned to their former positions in a few seconds after removing the force. During the last 5 minutes of experiment 14 the magnetic force which stressed the folds was removed and then restored by stages, while the corresponding positions of the folds were observed and plotted (Text-fig. 3). These results do not lead to a value for the elastic modulus of the tissues, because it seems impossible to define what is being stressed without ambiguity.

All the experiments (with the exception of experiments 5, 11, and 12) were performed upon embryos which were normal and quite undamaged in any way before the experiment, during the experiment, or after the experiment. The wax depression which supported the neurulae during the experiments was shaped to the contours of the ventral half of the embryo so that it was not flattened. The weight of the dumb-bells themselves was easily supported by the neural plate without obvious flattening. When the dumb-bells were removed at the end of the experiment, the imprint of the ball-shaped ends of the dumb-bells was left on the neural folds, which were slightly retarded by the experiment, but no damaged cells were seen. A few hours later the folds had closed normally along their entire length and no sign of the experiment was visible.

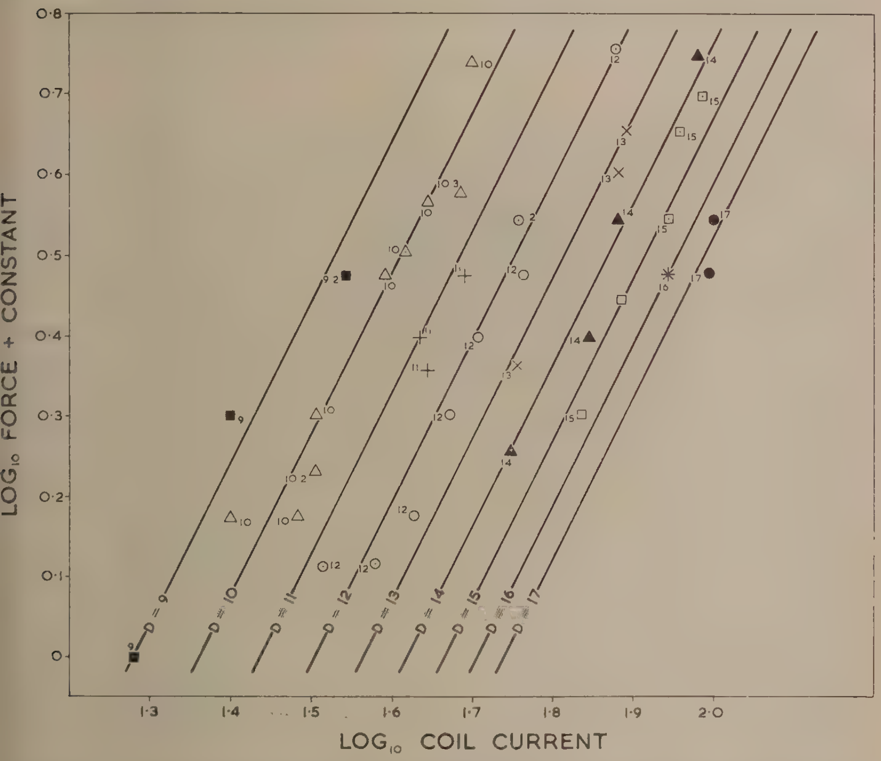
Experiments 5, 11, and 12 were made upon neurulae having wounds in the side of the embryo outside the neural folds but in line with the dumb-bells. Neurulation forces of only 10^{-2} dynes and less were recorded, although ample time was allowed for the experiment. Neural closure did, however, take place slowly when the inducing current was switched off altogether. These observations seem to suggest that the greater part of the force which the neural folds can exert is transmitted through the ventral ectoderm, and not as a result of a rolling-up movement of the neural plate itself. However, there are obvious dangers in interpreting results obtained on damaged embryos. It is not known how far-reaching were the effects of the wounds; nor were the effects of the healing reactions upon the measurements known. Moreover, it would have been better to have measured a normal neural closure force on an undamaged embryo, and then to have made the experiment on the same embryo after wounding, which would at least have proved the normality of the embryo at the beginning of the experiment. The work of previous authors (Glaser, 1914; Boerema, 1929; Brown, Hamburger, & Schmitt, 1941; and Holtfreter, 1944) strongly supports the conclusion that the capacity for folding and the closure of the neural tube is intrinsic in the tissues of the neural plate itself. The presence of other tissues is not necessary for neural closure. It is not known, however, in what degree the other tissues, in particular the lateral and ventral ectoderm, help or hinder the folding and closure of the neural tube.

Direct calibration

The forces of mutual repulsion between the dumb-bell pairs were calibrated against a quartz-fibre torsion-balance which was constructed like that illustrated on p. 215 of Strong's *Modern Physical Laboratory Practice*. The various ancillary techniques for handling quartz fibres which were used in its construction were those described by Nehr (1938) in the same book. The torsion-balance was calibrated, with its quartz cross-arm in the horizontal position, by weighing a series of microweights of between 50 and 100 μg., each prepared from known lengths of tungsten wire of 0·0005 in. diameter, 51·4 cm. of which had previously been found to weigh 1·393 mg. The torsion-balance was found to have a sensitivity of 3·85 μg. per degree deflexion of the cross-arm, when the weighed object was fixed 4·6 cm. from the torsion fibre. When it was used with the cross-arm vertical and observed under the conditions of the measurements described below, it could measure forces to a quarter of a microgramme weight (i.e. to an accuracy of about $0{\cdot}25 \times 10^{-3}$ dynes).

Throughout the measurements by which the magnetically applied forces used in the experiments on neural closure were evaluated, the dumb-bells were mounted in precisely the same position with respect to the electromagnetic coils as in the illustration of Text-fig. 2, but while one of the dumb-bells was mounted on a strong rigid quartz support, the other was mounted at the tip of the quartz cross-arm which was in the vertical position of equilibrium. The cross-arm was 4·6 cm. long from dumb-bell to torsion fibre. The perspex box, which contained the embryo in the main experiments, was of course absent, and the dumb-bells were surrounded by air. The dumb-bells were fixed to the quartz fibres by minute amounts of sealing-wax with the aid of an electrically warmed wire loop. A small correction was applied for the weight of the sealing-wax. The dumb-bells were sealed so as to remain always parallel to each other and in the same horizontal plane, but while one dumb-bell was immovable the other was free to swing away from its partner when a current passed through the coils. The pair of electromagnetic coils were correctly positioned on an adjustable cradle which was clamped across the top of the draught-proof perspex case which contained the torsion-balance. A plane glass window was let into the box immediately above the dumb-bells, so that they could be viewed by the microscope with a scale in the ocular to record the position of each dumb-bell and the separation between them. The equilibrium position of the movable dumb-bell for zero current and therefore for zero torsion in the quartz system was first noted. Then when a known current was passed through the coils, this dumb-bell moved away from its partner and came to rest in a new equilibrium position, when the magnetic force of mutual repulsion was equal to the opposing couple applied by the torsion in the horizontal quartz fibre. From a knowledge of the previously measured torsion constant of the balance, the magnetic repulsion force between the dumb-bells was then easily calculated for the particular value of the current through the coils and the particular separation between the dumb-bells. By making a small measured alteration to the position of zero equilibrium, it was then possible to record a new value for the magnetic repulsion force corresponding to a new current value, but with the same separation between the dumb-bells. Then the magnetic repulsion force for the same current value, but a different separation, was recorded. In this way the forces of neural closure were, in effect at least, directly calibrated by a method of weighing.

Some small corrections were applied to the readings of the torsion-balance when it was used with its cross-arm in the vertical position, using a graphical calibration procedure. The corrections were for a small amount of sag in the torsion fibre and for the resolved part of the weight of the movable dumb-bell tending to deflect the balance whenever the cross-arm was not exactly vertical and adding slightly to the deflexion caused by the magnetic repulsion between the dumb-bells. The exact values of these corrections varied with the mass of each dumb-bell.



TEXT-FIG. 5. The graph shows the results of the direct calibration carried out with the particular dumb-bell pair which was used for experiment 14. The straight lines are each for particular values of separation D between the dumb-bells measured in scale-divisions of the microscope ocular (10 scale-divisions = 300 μ). The experimental points carry alongside them their particular value of separation in scale-divisions.

For each pair of dumb-bells a plot of the magnetic repulsion force F against coil current i gave a family of parabolas with one parabola for each particular value of the separation D between the dumb-bells, each parabola having a common apex at the origin and a common force axis. This showed that the mutual repulsion force was proportional to the square of the coil current. A plot of log F against log i therefore gave a series of straight lines of slope 2, each for a particular value of D (Text-fig. 5). Using this graph, the forces of mutual repulsion between the dumb-bells, which had been noted down during the neurulation experiments only in terms of the current i and separation D, could then be evaluated in dynes using the measured balance constants. In this way repulsion forces between eight separate pairs of matching dumb-bells having lengths and masses distributed evenly over the entire range used in the neurulation experiments were calibrated directly. Five of these pairs were used in actual neurulation experiments, and the values recorded for the forces of neural closure in these experiments are estimated to be accurate to about 5 per cent.

Indirect calibration

The forces of magnetic repulsion between dumb-bells in another nine experiments had to be indirectly calibrated because of the loss or damage to dumb-bells caused by accidents when handling these small objects, which occurred between a neural closure experiment and the completion of a direct calibration A method for doing this was devised and the results obtained in this way are dependent upon the results of the direct calibrations with other dumb-bell pairs

All the eight dumb-bell pairs which had been used for direct calibration, and such of the others as remained whole, were weighed to a microgramme using the torsion-balance. All the dumb-bells used in the neurulation experiments had been previously sketched and carefully measured under a microscope. These measurements included the three principal diameters of each end of each dumb-bell, the length and average diameter of the shaft, and the total length. From these measurements their masses were calculated, and agreement to 7 per cent was achieved with the results of the weighings.

The forces of mutual repulsion between dumb-bell pairs for particular values of separation *d* and coil current *i* were roughly proportional to the square of the mass *w* of the dumb-bell pair or the sixth power of the length *L*, but pairs of identical length gave forces which were greater according as they were the more massive. Using the data from the eight dumb-bell pairs which had been directly calibrated, a plot of log *w* against log *L* was made and the best straight line of slope 3 was constructed. Then using the same data, log *F* was plotted against log *L* for particular constant values of separation *d* and coil current and the best straight line of slope 6 was constructed. The deviation of each point representing a particular matching pair of dumb-bells, was then measured as the perpendicular distance from the point on to the best straight line. This was done for both the above graphs, taking the deviations as positive or negative according as they were on the side of the line representing dumb-bell pairs which were respectively more or less massive than the average. When the deviations from the above two graphs were plotted against each other, a third straight line was obtained giving a good fit to the points. Then for any other dumb-bell pair of known mass and length, these three graphs gave a value for the force of mutual repulsion between the dumb-bells for the same particular values of separation and current *i*. The value of the repulsion force for different values of separation was found from a further family of curves, which was plotted, of the increments of log *F* at constant coil current for other separations, again using data from the dumb-bells which were directly calibrated. The value of the force for the other values of coil current was easily found, because an accurate dependence upon the square of the current could be assumed. The forces of neurulation, in the nine experiments where direct calibration was not possible, were evaluated indirectly in this manner to give values of an estimated accuracy of 15 per cent., which were in agreement with the results of those experiments which were calibrated directly.

DISCUSSION

According to the present author's knowledge of the literature, two previous measurements of forces exerted by embryonic tissues have been made. Waddington (1939) measured the magnetic force required to prevent movement of a steel sphere of 83 μ diameter embedded among cells of a gastrula of *Triturus alpestris*. He found that the maximum force exerted by the gastrulating tissues was equivalent to 0·34 mg./mm.^{2} of the hemispherical surface of the steel sphere. This means that the gastrulating tissue exerted a force of $3{\cdot}6 \times 10^{-3}$ dynes on the embedded sphere. When the present author tried to perform a similar experiment, he observed that while a magnetic force was applied to a ball sufficient to hold it stationary, the gastrulating cells seemed still able to move round on either side of the ball, and in this way gastrulation continued. If these should have been the conditions under which Waddington's measurement was made, the maximum force which the gastrulating tissues were able to exert might be greater by anything up to a factor of say 10 times. In any case, Waddington's figure is of a similar magnitude to the present result for the maximum force which the neural tissue of the same species can exert, i.e. 40 to 45×10^{-3} dynes. Moore (1941, 1945) found that the gastrulation of the sand-dollar *Dendraster excentricus* was prevented by the presence of sucrose of osmotic pressure 0·37 to 0·75 atmospheres in the blastocoel cavity. Taking the surface area of the invaginating cone of the gastrula as 707 μ^2, Moore calculated that the necessary force to stop gastrulation in *Dendraster* was 2·6 to 5·5 dynes. (Moore actually calls this last figure a pressure, although he records it in milligrammes and clearly understands it as a force.) As Moore points out, his result is relatively enormous when compared with the measurements on newt, although the difference is not quite as great as Moore says it is.

If we take the rate at which neural closure of *Triturus alpestris* can take place at 20° C. as 200 μ/hour, against an externally applied force 40×10^{-3} dynes, then the maximum possible rate at which a neurula can perform work against an externally applied system is 8×10^{-4} ergs/hour or $1{\cdot}9 \times 10^{-11}$ calories/hour. During the whole neural closure process the work which may be so done is about 8×10^{-3} ergs. Tuft (personal communication) has measured the respiration of *T. alpestris* embryos. At 25° C. he found neurulae respired between 0·3 and 0·4 cu. mm. of oxygen each hour. If we take the respiration at 20° C. during neural closure to be 0·25 mm.^{3} of oxygen per hour and assume a calorific value of $4{\cdot}6 \times 10^{-3}$ calories per mm.^{3} of oxygen consumed, then we know the total energy available to a neurula is about $1{\cdot}15 \times 10^{-3}$ calories per hour. Assuming that metabolic energy is utilized by the neural closure process at an efficiency of 10 per cent., we find that the work which can be done by the folds of the neurula is about six million times less than the total metabolic energy available to the whole embryo. From the curve which Løvtrup (1953) published for the oxygen consumption of embryos of axolotl developing at 20° C., the oxygen consumed by an axolotl embryo at neural closure can be taken as about 0·45 mm.^{3} of oxygen per hour. Assuming a rate of closure of 200 μ/hour to be just possible against a force of 80×10^{-3} dynes, a similar calculation for axolotl again shows that the ratio of energy available from respiration to the maximum work which can be performed by the embryo is just under 6×10^6.

If we take the result of Waddington (1939) that the gastrulating cells of *Triturus* were shown to exert a force of $3{\cdot}6 \times 10^{-3}$ dynes, then assuming the tissues invaginated at a rate of 125 μ/hour, the gastrula was known to work at a rate of $4{\cdot}5 \times 10^{-5}$ ergs/hour or about 10^{-12} calories/hour. If we assume that the gastrula respired 0·1 mm.^{3} of oxygen per hour at the room temperature of the experiment, then the total energy available to the embryo was about $4{\cdot}6 \times 10^{-4}$ calories/hour, and assuming an efficiency of 10 per cent. for the gastrulation process we find the ratio of total energy available to the energy which can be used for doing work in gastrulation is about 5×10^7. If the argument is accepted that a gastrula is capable of exerting a force up to 10 times greater than Waddington's figure then the ratio agrees with the corresponding one calculated from the neurulation experiment.

Moore (1941, 1945) calculated for *Dendraster* that the energy available to this embryo from its respiration was 2,000 times greater than that which was used in gastrulation under the conditions of his experiment. This is, of course, a very different figure from that which has been calculated above for morphogenetic processes in amphibian embryos, but the mechanical work which the tissues can perform is still a minute fraction of the total energy available.

It is important to remember that all the experimental measurements of morphogenetic forces, whether made by this or other authors, are measurements of forces developed by embryos against an outside system introduced by the experimenter. They are not and cannot be measurements of the forces which an embryo normally exerts against its own tissues in order to bring about gastrulation or neurulation under normal conditions. When a morphogenetic movement is just brought to rest by applying an opposing force, the magnitude of the opposing force gives a measure of the force which the embryo can exert in excess of the force which it already is exerting against its own elastic and other physical properties. Therefore the estimates of work done derived from such experiments refer to the work which may be performed by the embryo in excess of that which it normally is called upon to perform. It would seem that the work performed in normal gastrulation and neurulation could only be calculated theoretically from a complete knowledge of the process and all the physical constants involved in it. In the following way, however, it may be argued that the physical work which the embryo normally performs to change the shape and mutual dispositions of the cells is likely to be of the same order of magnitude as that which it can be shown to be capable of performing against an external system. The gastrulation and neurulation processes have evolved in such a manner that they can be successfully completed over a limited range of temperature and other environmental conditions, all of which must alter the physical properties of the cells themselves and therefore the work required to change their shapes. No quantitative estimates of the effect of temperature on the physical properties of amphibian cells are available, and one is forced to seek analogies elsewhere. For instance, Heilbrunn (1930) has shown that the 'plasm viscosity' of an amoeba varies in a complicated manner with temperature, and can take values which differ by a factor seven within a range of less than 10° C. Mitchison & Swann (1954) measured an increase in the stiffness of the cortex of unfertilized sea-urchin eggs of times 2·1 for a temperature drop of 18·5° C. Alfrey (1948) quotes many examples of polymer systems whose stiffness varies sixfold over 10° C.

It is not unreasonable, therefore, to suggest that embryos at less favourable temperatures must perform in morphogenesis several times the work that is needed at the most favourable temperature. On the other hand, it is unlikely that in newt or axolotl a gastrulation or neurulation process can have been evolved that is capable of exerting closure forces vastly in excess of that which is ever needed. It seems more likely that the tissues can normally exert a few times the force which is necessary, and this view is supported by the following tentative derivation of the work normally done by a newt neurula in closure. Without having to accept any particular theory of neural closure, a number of which are briefly discussed by Brown, Hamburger, & Schmitt (1941), it may be supposed that however the cells of the neural ectoderm become progressively more wedge-shaped, so that the plate becomes a tube, some work must be done against elastic forces. If the movements towards closure are pictured as taking place in little steps, during each one of which work is done in straining the cells slightly, then during the interval between each step of deformation we may suppose that the stresses have time to become completely relaxed, partly because the cells have plastic as well as elastic properties and partly because the cells can rearrange themselves slightly and take up positions of least strain within the tissue. Under the conditions of the present experiment, stepwise closure was observed and the steps were roughly of the order 13 μ, which happened to be just the distance moved in each of three closure movements in experiment 14 for example (see Table 1). From the present experiments a 13 μ deformation of the neural folds is known to be produced by a force of about 10^{-2} dynes, and the work required to produce it is the product of one-half this force times this final deformation, which gives $6{\cdot}5 \times 10^{-6}$ ergs, so that complete closure, which would demand about 154 of these steps, requires 10^{-3} ergs, which is a rate of working of about

10^{-4} ergs/hour. So this very rough estimate for the work which the neurula does against its own elastic properties at 20° C. gives a figure which is an eighth part of the previously calculated maximum working rate against an external system.

SUMMARY

1. It is argued that an estimate of the fraction of the total energy available to an embryo which is necessary to achieve gastrulation or neurulation can only be valid when based upon measured forces exerted by embryos.

2. A method is described for measuring the forces which the neural folds can exert towards closure. A pair of dumb-bell-shaped electromagnetically induced magnets was placed on the neural plate against the neural folds. The force of mutual repulsion between the magnets was balanced against the closure force exerted by the embryo so that values for the latter were obtained.

3. The elasticity of the neural fold tissues was demonstrated.

4. Under the conditions of the experiment, neural closure movements took place between intervals of no movement. The force, which an embryo exerted, increased with time to a maximum value.

5. Neurulae of *Triturus alpestris* exerted forces up to between 40×10^{-3} and 45×10^{-3} dynes. Neurulae of axolotl, *Siredon mexicanum*, exerted up to between 90×10^{-3} and 110×10^{-3} dynes.

6. During the experiments with neurulae the repulsion forces between the dumb-bells were noted in terms of the separation between the dumb-bells and the current in the magnetizing field coils. The corresponding forces were evaluated in separate calibration experiments with the same dumb-bells by a method of weighing using a sensitive quartz-fibre torsion-balance. In other cases, calibration was indirect but based upon the measurements obtained in direct calibration.

7. The present results are shown to be of similar magnitude to those of Waddington (1939) for gastrulae of the same species. Calculations show that newt and axolotl neurulae can only do work in closure against an external system at a rate which is six million times less than the total energy used by the embryo. *Triturus alpestris* neurulae can work at 8×10^{-4} ergs/hour at 20° C. Another more tentative calculation suggests that the work which a newt neurula normally does against the elastic properties of its own tissues is about an eighth of this.

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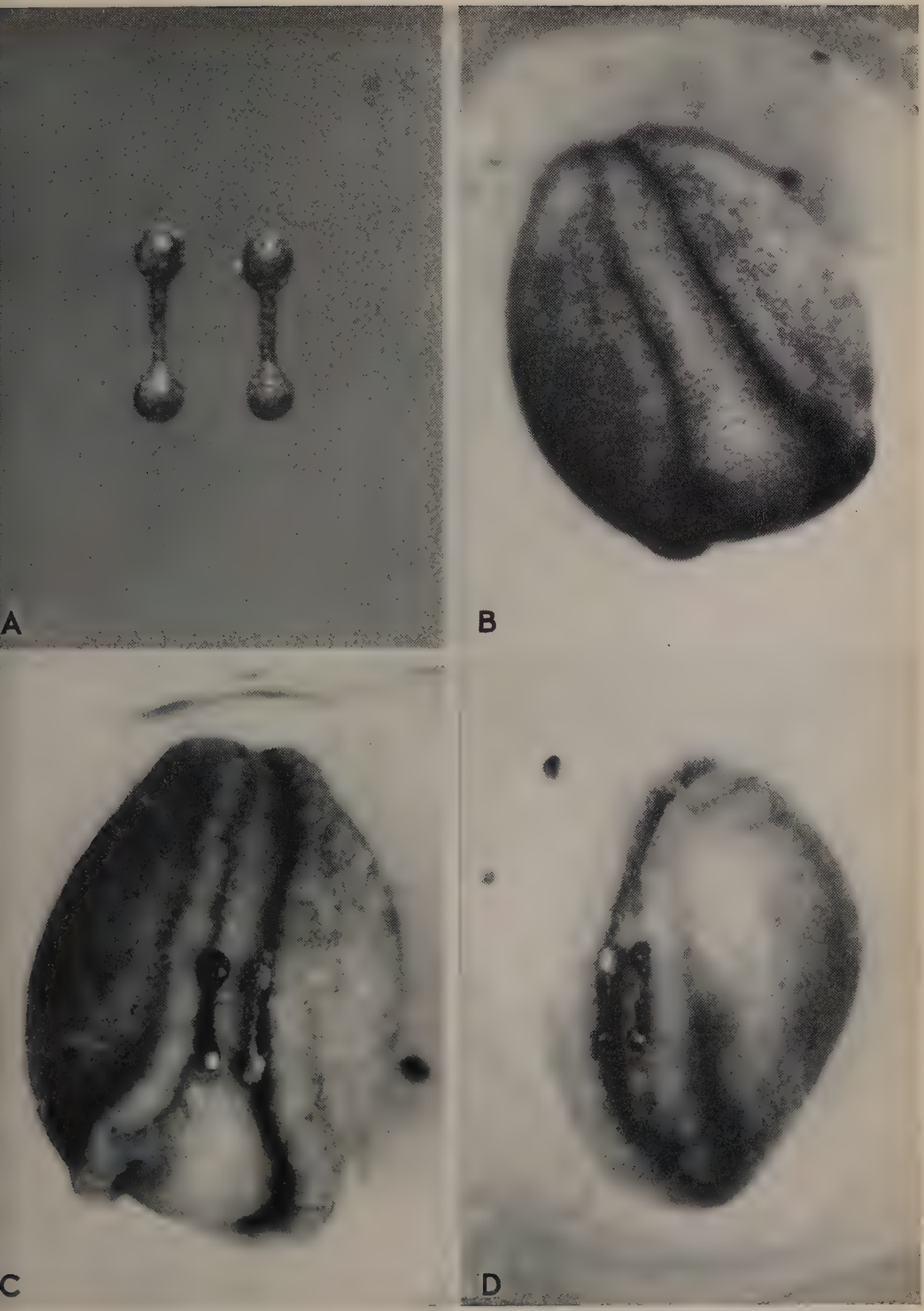

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EXPLANATION OF PLATE

FIG. A. A typical pair of dumb-bell-shaped induced-magnets used in these experiments.

FIG. B. An axolotl neurula at the stage chosen to begin an experiment on neural closure in the mid-fold region.

FIG. C. An axolotl neurula with a dumb-bell pair in position on the neural plate against the neural folds, in the mid-fold region, at the beginning of an experiment on neural closure.

FIG. D. An axolotl with a pair of dumb-bells in position against the neural fold towards the head region. The embryo is tilted so that the dumb-bells are horizontal.

(Manuscript received 10 : ii : 58)

The Mode of Growth of the Tail in Urodele Larvae

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WITH TWO PLATES

INTRODUCTION

THE idea that the hinder part of the trunk together with the tail or the tail alone develops by the outgrowth of a cell mass which is in every respect indifferent has been disproved since 1928 for the Amphibia. The results of experiments with vital staining (Bijtel & Woerdeman, 1928; Bijtel, 1929, 1931) and with microsurgical methods (Bijtel, 1936) have shown that the presumptive rudiments of the tail organs (epidermis, spinal cord, muscle segments, tail-gut) are already present in the neural plate stage as more or less separate cell territories. During and immediately after the transformation of the neural plate into the neural tube, these cell territories are brought together into the tail-bud by morphogenetic movements. Holmdahl (1939 *a*, *b*, 1947) and Vogt (1939) have criticized this conception. They adhered to the view that the organs of the hinder part of the trunk and of the tail (Holmdahl) or only the axial organs of the tail (Vogt, p. 127) originate from an indifferent blastema. The results of experiments published since 1936 by other investigators, however, have confirmed that there exists no specific difference between the mode of development of the anterior part of the body and that of the posterior part (Nakamura, 1938 *a*, *b*, 1942, 1947, 1952; Pasteels, 1939 *a*, *b*, 1943; Suzuki & Kobayashi, 1939; Suzuki, 1940; Spofford, 1945; Chuang, 1947; Ford, 1950).

In the neural plate stage of urodele larvae the arrangement of the component parts of the tail-bud is most remarkable. The presumptive material for the tail somites occupies the hindmost part of the neural plate. This material does not invaginate, but is continuous with the already invaginated chorda-mesoderm round the dorsal and lateral blastopore lips. The caudal end of the material for the spinal cord, i.e. the presumptive tip of the tail, is to be found in the neural plate at some distance in front of the blastopore. At this level the caudal extremity of the tail somite material is continuous with the extreme limit of the tail neural material. In the developing tail-bud the prospective mesoderm folds under the notochord and continues into the tail neural tube round the tip of the noto-

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chord. Sagittal sections through the outgrowing larval tail show this connexion (Bijtel, 1931, figs. 14, 17).

In view of the remarkable arrangement of the presumptive material for the organs of the tail, the regulative capacities, the rate of differentiation, and the inductive potencies of the hinder part of the neural plate have been investigated: the experiments of Münch (1938), von Aufsesz (1941), Chuang (1947), and ter Horst (1948) may be mentioned. The results of all these investigations confirm that the posterior part of the neural plate in Urodeles has a mesodermal significance, though its position would suggest an ectodermal one. In grafting experiments between Nile-blue stained embryos and unstained embryos of Amblystoma, Spofford (1945) has determined with great exactness the prospective potencies of this mesoderm. He has found that a variety of mesodermal structures are of 'neural origin', including the myotomes of the posterior third of the trunk.

Doubt has existed as to whether the material for the notochord invaginates entirely in the neural plate stage or not. In his last study on the development of the tail Vogt (1939, p. 122) denied the presence of mesoderm in the posterior part of the neural plate of Urodeles and stated that the extreme limit of the neural material is connected with that of the notochord. According to him the caudal tip of the notochord lies in the dorsal rim of the just closed blastopore and even partly on the surface in the neural plate. The investigations mentioned above exclude, however, any contact between the material for the notochord and that for the neural tube. The results of the extirpation experiments of von Aufsesz (1941) and of Chuang (1947) have confirmed my view (1929) that the caudal extremity of the notochord anlage does not lie on the surface in the wide open neural plate stage, but has already entirely invaginated and lies in the gut roof. It has not yet been exactly determined how far from the dorsal rim of the blastopore the extreme limit of this organ lies just before the closure of the neural tube. The possibility that in this stage its extreme limit is at the level of the extremity of the neural material in the overlying ectodermal layer cannot be excluded. This needs further examination.

Though a great number of observations are available concerning the arrangement of the anlagen of the tail organs in the neural plate stage and on the way in which they are combined in the tail-bud, little is known about the mode of growth of the tail, which obviously cannot be treated apart from the growth in length of the rest of the body.

It is a well-known fact that the notochord plays an important part in the axial stretching of the body. Kitchin (1938, 1949), Hörstadius (1944), and Nieuwkoop (1946), who removed parts of the invaginated notochord rudiment, either just before or at the neural plate stage, report an extreme shortening of those parts of the body in which the notochord is missing. The same observation has been made by Lehmann & Ris (1938), who suppressed notochord development with lithium chloride. These larvae, which could be reared until development of the hindlimbs, had a characteristically thick-set shape, ascribable to the deficiency of the notochord combined with a considerable shortening of the myotomes. Though these larvae resemble in various anatomical details larvae from which the notochord has been operatively removed, the possibility cannot be excluded that other tissues than the notochord are affected by the lithium chloride. Lehmann himself has shown (1933, 1934) that musculature and intestine are relatively normal in the lithium-treated larvae, whereas the nervous system shows a great reduction in consequence of the reduction of the organizer. A shortening of the myotomes and often also a fusion of the myotomes of the two sides is reported by all those who have studied larvae with a deficiency of the notochord.

The question whether the notochord plays an active part in the growth of the body, or only acts as a supporting organ during the growth of the other tissues, has not been solved. Bytinski-Salz (1936), Holtfreter (1939), Chuang (1947), and others have established that during its development the notochord has the power of autonomous elongation. In the absence of myotomes and spinal cord, however, its course is described as coiled and it is often compressed in a craniocaudal direction. Mookerjee, Deuchar, & Waddington (1953) and Mookerjee (1953) have called attention to the fact that it is the notochordal sheath which plays an important role in the mechanism of elongation of the notochord. According to Mookerjee the elongation of the notochord seems to be a direct result of its confinement within the relatively inelastic sheath, which is laid down by surrounding extra-chordal mesoblastic cells (Holtfreter, 1939; Mookerjee, 1953). The notochordal sheath in this way converts the increase in volume of the notochord into a growth in length.

Pasteels (1939*b*) and with him Dalcq (1941) have seen in the notochord the principal 'motor' for the outgrowth of the tail, though they ascribe an important function in this process to the neural tube and the myotomes as well. Devillers (1948), however, has concluded from extirpation experiments in the trout that at least in early stages elongation of the body can take place independently of the notochord.

Bytinski-Salz (1936) gives the principal role in the process of tail-growth to the somite material. In this connexion it is worth mentioning that in tissue cultures of the isolated amphibian somites the myoblasts can grow lengthways and differentiate into long, mostly unbranched, muscle cells (Holtfreter, 1939). The same cells have been found in tissue cultures of the hinder part of the neural plate of the axolotl and of *Triton taeniatus* (Bijtel, 1936).

The results of my former experiments have given me the impression that the notochord is not the principal 'motor' in the process of the growth of the tail, but that on the contrary the neural tube with the somites play the most active part, whereas the notochord functions as a splint. In recent years I have tried to complete the series of experiments. The results, which will be described here, have strengthened me in my conviction.

EXPERIMENTAL PROCEDURES

Six series of operations concerned with the potencies of the hinder part of the neural plate and the hinder part of the notochord have been performed on larvae of the axolotl (*Siredon mexicanum*) and of *T. taeniatus* and *T. alpestris*. The larvae of the axolotl are best suited to these experiments, since it is easier to loosen the hinder part of their neural plate from the gut roof than in the *Triton* species.

The larvae were preferably operated upon in stages with wide open neural plate, viz. those of axolotl in stages 14, 15, 16 (normal table of Harrison), those of *T. taeniatus* and *alpestris* in stage 14 (normal table of Glaesner). A small number of larvae has been used in older stages.

The operations were performed as far as possible under sterile conditions as indicated by Woerdeman (1930). They were done in sterile Holtfreter's solution. Immediately after, this was largely replaced by filtered tap-water, which promotes wound healing.

The larvae were reared, if possible, until about stages 41–42 (Harrison, Glaesner). They were then fixed in Bouin's fluid, sectioned at 10 μ, and stained with haematoxylin-erythrosin or with azan.

EXPERIMENTAL RESULTS

The following six series of experiments have been performed.

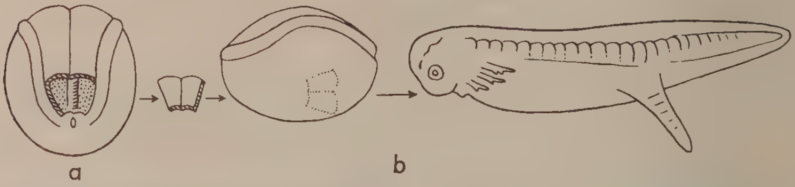
1. Extirpation of the hinder part of the neural plate, containing the material for the tail neural tube and the tail somites.
2. Transplantation of the same part of the neural plate into the lateral side of another larva.
3. Extirpation of the hinder part of the notochord.
4. Transplantation of the hinder part of the notochord into the lateral side of another larva.
5. Rotation through 180° of the hinder part of the neural plate, containing the material for the tail neural tube and the tail somites.
6. Rotation through 180° of the hinder part of the notochord.

1. *Extirpation of the hinder part of the neural plate, containing the material for the tail neural tube and the tail somites* (Text-fig. 1*a*)

This material was removed in order to test the potency of the tail notochord to grow out *in situ* and to produce a tail in the absence of tail neural tube and tail mesoderm.

Operations of this kind cause big wounds, which often close badly and give rise to fistulae with severe malformations on the dorsal side of the growing larvae. In a number of larvae, however, the wounds closed well. Only these last have been used to judge the effect of the operation. The effect is that the tail is totally missing or is present in a more or less reduced form. The earlier the operation takes place, the more frequently is the tail absent or strongly reduced.

Embryo Mes. E. 3a, Axolotl. In stage 15 a rectangular piece of the neural plate without underlying material was removed. It extended from the blastopore forwards and consisted of about one-fourth of the total length of the neural plate. The wound healed well. Outwardly a stunted tail developed, curved ventralwards (Plate 1, fig. A).



TEXT-FIG. 1. Extirpation and transplantation of the hinder part of the neural plate, containing the material for the tail neural tube and the tail somites. *a*, donor; *b*, host.

This larva was reared until the gills and the limb-buds had the appearance of about stage 42. Sections show that myotome material, though arranged irregularly, is still present in the base of the tail. The rudimentary tail consists of a notochord accompanied by a thin neural tube (Plate 1, fig. B). The notochord shows signs of incomplete growth. It has not stretched well and its contour is irregular. Moreover, it shows some retardation in its histological differentiation, expressed in an incomplete transformation of the yolk and a defective vacuolization. The notochord sheath seems to be defective in places.

Other larvae operated upon in the same way have a similar appearance. The tail is either almost absent or very small. The more the neural tube and the somites are defective, the more stunted is the tail. Consequently, the larger the piece of the neural plate removed, the smaller the tail rudiment that develops.

In the same way the malformation is more severe when the larva has been operated upon at an earlier stage. In the early neural plate stage, invagination of presumptive material for the trunk somites is still taking place and this material lies on the surface in the neural plate. Consequently, operation at this stage produces a greater defect in the somites.

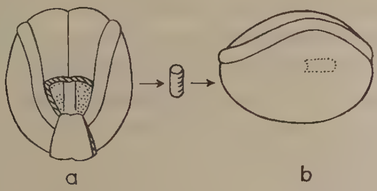
2. *Transplantation of the hinder part of the neural plate, containing the material for the tail neural tube and the tail somites into the lateral side of another larva* (Text-fig. 1*b*)

In order to test the growth potencies of the tail neural tube and the tail somites, the material of the hinder part of the neural plate, which was removed in the first series of experiments, was transplanted into the lateral side of another larva. A transplant of this kind behaves as a growing tail.

Embryo AX. 23, Axolotl. From an axolotl larva in stage 15 a rectangular piece of the hinder part of the neural plate was transplanted without underlying material into the ectoderm of the lateral side of another larva of about the same stage. The grafted piece extended from the blastopore forwards and consisted of about one fourth of the total length of the neural plate. The lateral margins of the transplant became joined, and a tube was formed, lying beneath the epidermis. A protuberance developed, in which the growing graft, covered by the epidermis, folded itself double at the border between neural and somite material. From this protuberance there grew out an appendage. It contained an axis, formed by the neural tube with somites. A longitudinal section through it shows the regular arrangement of the somites along the neural tube (Plate 1, fig. C). In Plate 1, fig. D the arrangement of the somite material with regard to the neural tube is shown in a transverse section of a corresponding appendage of *T. alpestris* host AX. 18.

3. *Extirpation of the hinder part of the notochord* (Text-fig. 2*a*)

The stretching potencies of the tail neural tube and the tail somites have been studied *in situ* by extirpating the hindpart of the notochord.



TEXT-FIG. 2. Extirpation and transplantation of the hinder part of the notochord. *a*, donor; *b*, host.

Embryo C.E. 80, Axolotl. The hinder part of the notochord anlage, not only that of the tail, but of part of the trunk as well, was removed at stage 15. A rectangular piece of the neural plate was loosened on three sides and turned towards the blastopore. The notochord, now visible, was removed up to the neighbourhood of the blastopore rim and the wound covered again with the neural plate.

In these circumstances the hinder part of the neural plate grew out and formed a tail with a neural tube and somites. The initial stretching of the operated larva was not retarded, and particularly at first the tail showed few points of difference from a normal one. In more advanced stages, however, the tail lagged in growth in comparison with the normal, though the trunk showed an even stronger reduction of growth in length than the tail (Plate 1, fig. E).

This larva was reared up to about stage 42. The arrangement of the organs of the tail showed no other abnormality than the absence of notochord (Plate 2, fig. F). The dorsal and ventral fins developed as usual and the spinal cord was flanked by the myotomes of both sides. The muscle cells ran in a longitudinal direction next to the epidermis.

4. *Transplantation of the tail notochord into the lateral side of another larva* (Text-fig. 2*b*)

In order to test the stretching capacity of the grafted notochord anlage, the pieces of the notochord rudiment removed in the preceding series of experiments were transplanted beneath the ectoderm into the lateral side of another larva in the neural plate stage.

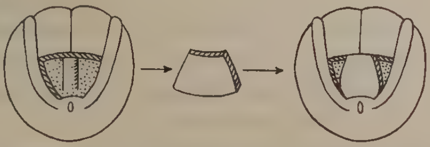
Embryo C.T. 14, Axolotl. A piece of the notochord anlage, containing the material for the hinder part of the trunk notochord and for the tail notochord, removed from larva C.E. 68, Axolotol, in stage 15, was grafted into the lateral side of another axolotl larva, C.T. 14, in stage 14 beneath the ectoderm. This host was fixed in stage 41. At the lateral side of the trunk the graft made a small protuberance in the epidermis. Transverse sections through the trunk show that the piece of notochord anlage has much increased in volume. Most of the notochord cells have lost their yolk and have vacuolized. The notochord is covered with a sheath. It has not, however, stretched straight, but has become coiled (Plate 2, fig. G).

In 1927 Woerdeman performed some experiments on larvae of axolotls, which throw some light on the problem of the direction in which the tail grows (Bijtel & Woerdeman, 1928). A large rectangular part of the neural plate together with the underlying gut roof was cut out, rotated through 180°, and replaced. The caudal edge of the graft was at some distance in front of the blastopore. A number of animals operated upon in this way developed in a very peculiar manner. They all had a rudimentary tail, while the rotated graft had developed into a tail process which had grown in a cranial direction. In this tail process the normal structures, neural tube, notochord, and axial mesoderm, had developed in a normal arrangement up to the very tip. In the tail proper only small myotomes and some mesenchyme were to be seen, with no neural tube and no notochord. It was evident that these rotation experiments were in perfect harmony with the results of my staining experiments, and at that time supported the conceptions based on these staining experiments.

In order to test whether it is the notochord, or the combination of the presumptive tissues of neural tube and somites, which determines the direction in which the tail grows, both factors were examined in the next two series of experiments.

5. *Rotation through 180° of the hinder part of the neural plate, containing the material for the tail neural tube and the tail somites* (Text-fig. 3)

Embryo Med. pl. D. 12, Axolotl. In stage 15 a large rectangular piece of the hinder part of the neural plate, extending to just in front of the dorsal lip of the blastopore, was removed without underlying chorda-mesoderm, rotated through 180°, and reimplanted at the same place. The graft healed well. As the larva grew up, a tail process developed at the dorsal side of the trunk in a cranial direction, whereas no tail appeared in the normal direction (Plate 2, fig. H). As might be expected, the heterotopic tail contained a neural tube and somites, just as did the grafts in the experiments of series 2, the spinal cord obviously lying on the posterior side of the tail.



TEXT-FIG. 3. Rotation through 180° of the hinder part of the neural plate, containing the material for the tail neural tube and the tail somites.

In other cases of this series, besides a dorsally or more cranially directed tail process, a small normally situated tail also developed, containing only myotomes and a slightly irregular notochord, but no spinal cord.

A remarkable fact is worth noticing. In various cases the heterotopic tail contained at its base a small notochord originating from the trunk notochord. A similar notochord has never been observed, when the same rectangular piece of neural plate, instead of being rotated and reimplanted at the same place, was grafted into the lateral side of another larva, as in the second series of experiments. In the next section this phenomenon will be discussed.

6. *Rotation through 180° of the hinder part of the notochord anlage*

Embryo C.O. 10, Axolotl. Though it is not very likely that the direction in which the tail grows should be influenced by the growth of the third axial component of the tail, nevertheless this possibility must be faced. For this reason, in embryo C.O. 10, Axolotl, the hinder part of the notochord anlage was excised, rotated through 180°, and reimplanted in the same place at stage 15. The tail grew out in the normal direction (Plate 2, fig. J). In none of the 12 larvae, operated upon in this way, has the tail grown in a cranial direction. As the extreme end of the rotated notochord implant and the surface of the amputated notochord anlage, however, had not always grown well together, at this place a slight curvature of the body, either in a dorsal or in a ventral direction, was in some cases to be seen.

DISCUSSION

Amongst the different parts of the body of urodele larvae the tail lends itself best to the study of the process of growth in length. The component parts of the tail can easily be isolated and studied separately and in varied combinations. The trunk in these larvae is less easy to study in this respect, as the gut loaded with yolk is a factor which interferes with rapid growth.

The results of the experiments of series 2 and 3 show that the hinder part of the neural plate, containing the material for the tail neural tube and the tail somites, can give rise to a tail-like appendage without notochord. If this material remains *in situ* after the removal of the notochord (series 3), or if it is grafted into the side of another larva (series 2), it folds along the border between neural material and somites and forms a tail process, growing out rapidly and consisting of a tail neural tube surrounded by tail somites. In the long run this tail process lacks the splinting action of the notochord; it finally becomes slack and bent.

If the part of the neural plate just mentioned is isolated, rotated through 180°, and reimplanted in the same place (series 5), a quite typical tail develops, which, however, grows in an abnormal direction.

The results of these 3 series of experiments support the view that the first growth of the tail-bud must be ascribed to the stretching potencies of the tail neural tube and especially of the tail somites. After removal of the tail notochord (series 3) the muscle fibres in the tail process are often seen running in a craniocaudal direction, attaching themselves to the epidermis for want of the notochord sheath. The results of these experiments show that the muscle fibres have the ability to grow and may reach a considerable length in the absence of the notochord.

Absence of the notochord in the trunk region of the body produces an extreme shortening of the trunk as described by various authors. In the experiments of series 3, I observed this malformation in those cases in which the notochord not only failed in the tail, but also in part of the trunk. Where the notochord is missing, and so is not interposed between the somites, the myotomes of both sides often fuse beneath the spinal cord. This phenomenon has been mentioned by various authors in larvae in which the notochord was removed surgically, and in those where the notochord development was suppressed by chemical means. In 1928 Woerdeman and I saw in fused myotomes, where the organs normally interposed between left and right myotomes (in this case neural tube and notochord) were missing, that the muscle fibres in a number of places had grown across the median line. The same phenomenon has now been observed in the trunk region and at the base of the tail of larvae in which myotomes had fused following notochordectomy. The muscle fibres here had often grown in a transverse direction instead of craniocaudally (Plate 2, fig. K). In consequence of the absence of the notochord these muscle fibres have obviously found the space to carry their intrinsic stretching potency into effect beneath the neural tube. From this it is evident that they are able to stretch provided they do not meet with too great a resistance from the surrounding tissues. In culture *in vitro* muscle fibres lengthen in the same way. It appears that they can also follow their stretching potency to a certain degree in a tail process, developing from the hinder part of the neural plate (experiments of the series 2, 3, and 5). In these three series of experiments the neural tube, which in itself has the power to stretch, functions as a preliminary splint. In the long run, however, another splint is necessary in the form of the notochord.

It is remarkable that in some communications dealing with fused myotomes in the absence of the notochord, muscle fibres with a transverse course are to be seen in the figures, though this is not mentioned by the authors (e.g. Lehmann, 1928, fig. 20; 1938, fig. 5; Münch, 1938, fig. 25; Muchmore, 1951, fig. 17). In a study on myotome fusion in the embryo of *Amblystoma punctatum* after treatment with lithium and other agents Cohen (1938), however, emphasizes the fact that the constituent cells of the fused myotomes interdigitate to produce the appearance of a continuous sheet. A similar movement of muscle cells has been observed in the development of ascidian tadpoles from isolated blastomeres (Cohen & Berrill, 1936). In this case the muscle cells of one side are lacking and those of the other side move into the unoccupied region.

As has been said (p. 472), the muscle fibres in the chordectomized tails are often seen running in the normal craniocaudal direction. In this part of the body the muscle fibres, it seems, are in a better position to follow their stretching potency in the normal direction than in the trunk, where the stretching power has to surmount the special resistance of the gut loaded with yolk. The experiments of series 2, 3, and 5 prove that the neural tube does not prevent the first growth in length of the muscle fibres, any more than does the epidermis. In the absence of notochord in the trunk, however, a number of muscle fibres cross the median plane and stretch beneath the neural tube. The more laterally situated muscle fibres remain short. They run in the normal craniocaudal direction and together with the transverse running fibres form the short myotomes, which give rise to the thick-set shape of these larvae.

This intrinsic stretching potency of the muscle fibres, which is already present at an early stage of development, is most probably bound up with the structure of the proteins of these fibres. Further investigations are necessary on this point.

After rotation through 180° of the hinder part of the neural plate containing the material of the tail neural tube and the tail somites (series 5), a small notochord originating from the trunk notochord has sometimes been observed at the base of the heterotopic tail (p. 473). It is out of the question that this small notochord was transplanted together with the piece of the neural plate. In the other grafting experiments of the hinder part of the neural plate notochord, tissue was never found in the outgrowing tail process. Spofford (1953) has made the same observation in corresponding experiments. In his investigations concerning the differentiation of the neural and mesodermal parts of the neural plate in *Amblystoma* Spofford has exchanged these parts with and without rotation between Nile-blue stained and unstained larvae, leaving the notochord rudiment intact. In some cases a small notochord of host origin was present in an ectopic tail exactly as in my cases. This small notochord at the base of the ectopic tail must have been induced by the surrounding tissues, most probably by the somites.

From the results of this series of experiments, in which the notochord was left unimpaired, it is clear that the material of the rotated graft, i.e. that for tail neural tube and tail somites, has determined the direction in which the ectopic tail, eventually with its induced notochord, has grown. Moreover, the results of the experiments of series 6 have shown that a rotation of the tail part of the notochord rudiment through 180° has no influence on the direction in which the tail grows. Finally, a piece of notochord transplanted into the side of another larva (series 4) does not give rise to a tail process, but only forms a coiled notochord covered with a sheath. Owing to its sheath a notochord has the power to elongate, as is shown by Mookerjee, Deuchar, & Waddington (1953) and Mookerjee (1953). The notochord rudiment, however, is moulded into a straight, slender structure only in the presence of the surrounding tissues, in which the somites, as has already been mentioned, most probably play the principal part.

SUMMARY

1. In the developing tail-bud of urodele larvae, the neural and the mesodermal components of the tail fold along their boundary and surround the caudal extremity of the notochord.

2. The first growth of the tail-bud is brought about by an autonomous lengthening of the neural and the mesodermal tissues.

3. The notochord does not have an active part in the process of the lengthening of the tail, but plays a passive role. It is a supporting organ, acting as a splint. In this way it determines the ultimate length of the tail.

4. It is not the notochord, but the combination of the neural and the mesodermal tissues, which in the first place determines the direction in which the tail grows.

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Extirpation of the hinder part of the neural plate. Embryo Mes. E. 3a, Axolotl. Stunted tail, curved ventrally.

FIG. B. Longitudinal section of the tail region of embryo Mes. E. 3a. *not.*, tail notochord; *nt.*, tail neural tube; *not'.*, notochord of the hinder part of the trunk; *nt'.*, neural tube of the hinder part of the trunk; *myot.*, myotome of the hinder part of the trunk; *cl.*, cloaca; *pron. d.*, dilated pronephric duct. ×67.

FIG. C. Longitudinal section of the tail-like appendage of embryo AX. 23, Axolotl. *nt.*, neural tube of the graft; *som.*, somite of the graft. ×65.

FIG. D. Cross-section of the tail-like appendage of embryo AX. 18, *Triton alpestris.* Abbreviations as in fig. C. ×200.

FIG. E. Extirpation of the hinder part of the notochord. Embryo C.E. 80, Axolotl. The length of trunk and tail is reduced.

PLATE 2

FIG. F. Cross-section of a notochordless tail. Embryo C.E. 80. *nt.*, neural tube; *myot.*, myotome with its cells lying alongside the epidermis. ×140.

FIG. G. Cross-section of a notochord graft. Embryo C.T. 14, Axolotl. *not.*, notochord graft; *not. sh.*, notochord sheath. ×95.

FIG. H. Rotation through 180° of the hinder part of the neural plate. Embryo Med. pl. D. 12, Axolotl. A tail process has developed in a cranial direction.

FIG. J. Rotation through 180° of the hinder part of the notochord. Embryo C.O. 10, Axolotl. The tail has grown in the normal direction.

FIG. K. Cross-section of the hinder part of the trunk of embryo C.E. 80 (fig. E). *myot.*, myotomes of both sides have fused. Transverse course of the muscle-cells. ×66.

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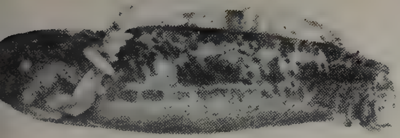

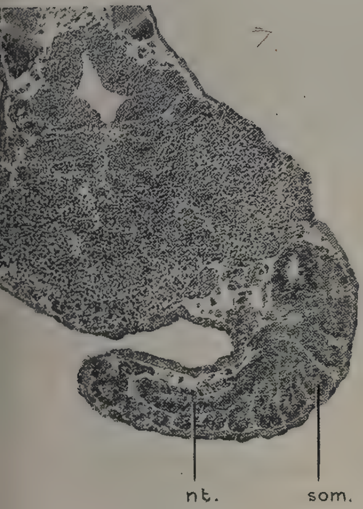

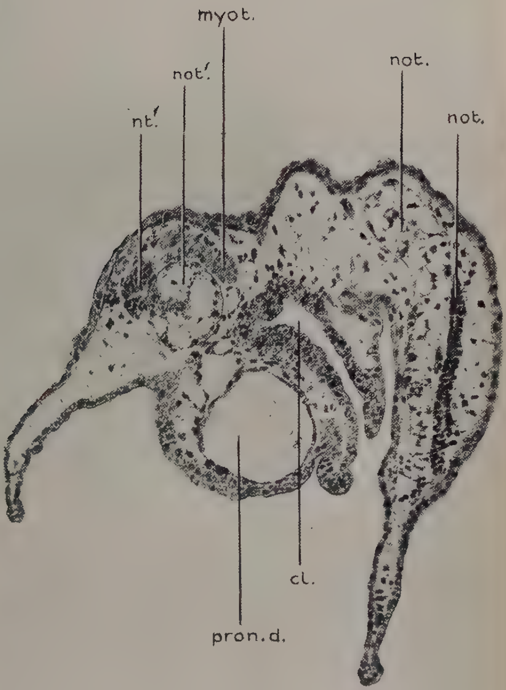

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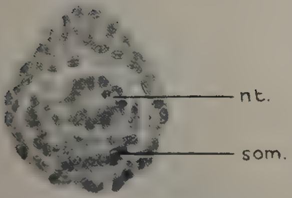

E

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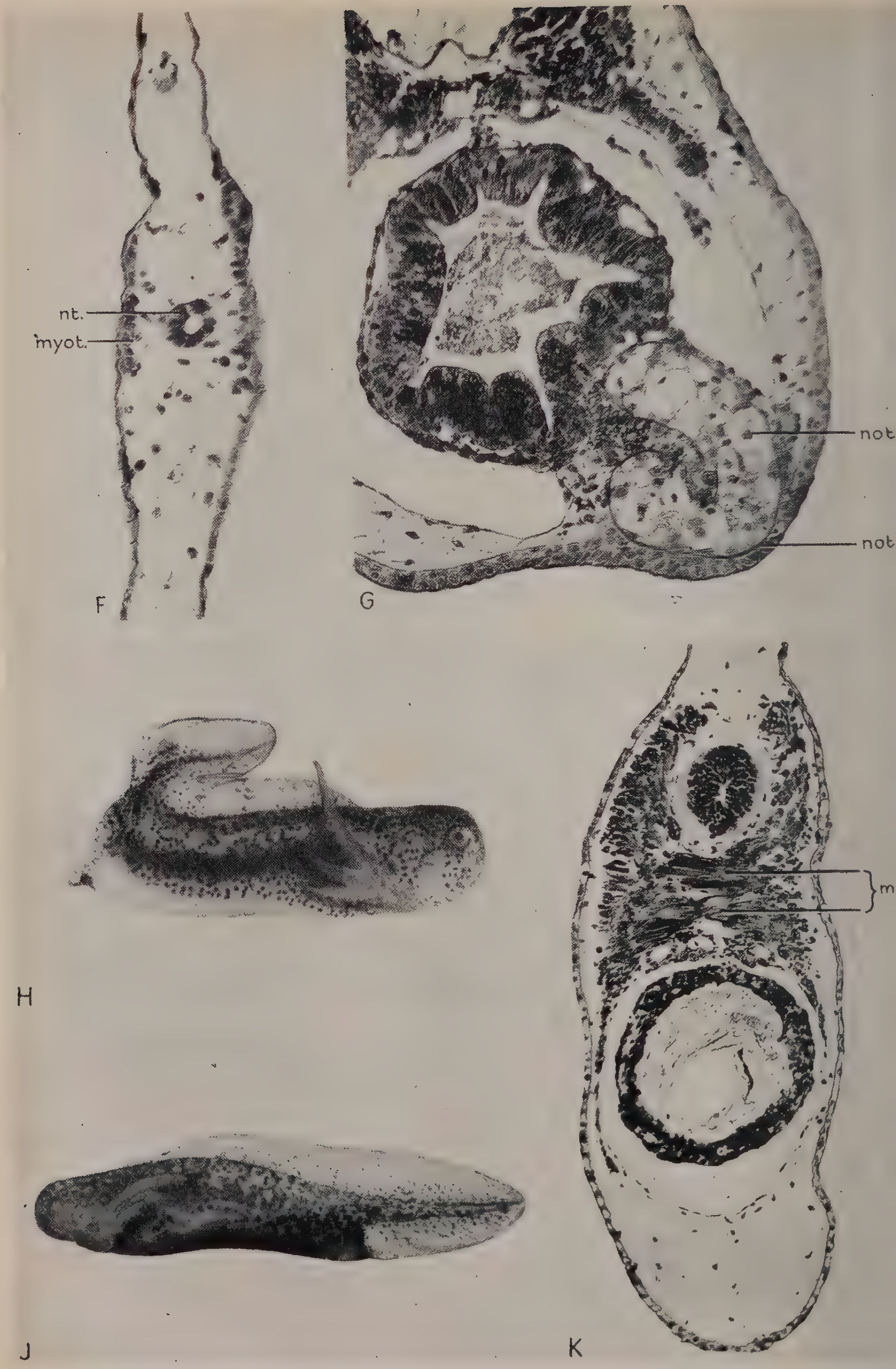
Plate 1



J. H. BIJTEL

Plate 2

The Dependence of the Cellular Transformation of the Competent Ectoderm on Temporal Relationships in the Induction Process

by SULO TOIVONEN[1]

From the Zoological Laboratory of the University of Helsinki

WITH ONE PLATE

In 1952, Nieuwkoop *et al.* suggested a new hypothesis concerning the induction phenomenon determining the early development of the amphibian embryo. This hypothesis was based on cleverly planned experiments in which folds of competent epidermis were transplanted on to different regions of the neural plate of the neurula. According to this hypothesis, the invaginating archenteron roof is supposed first to activate the overlying ectoderm, enabling it to develop autonomously to archencephalon and its derivatives. This same archenteron roof is later thought to exert a second effect, which they called transformation. This second action is considered responsible for modifying the differentiation tendencies of the activated archencephalon so as to result in structures typical of more caudal regions of the nervous system. This process is regarded as a quantitative one, so that with increasing strength of transformation, the differentiation tendencies would be progressively more caudal.

Later, Nieuwkoop & van Nigtevecht (1954) tried to obtain further evidence for this activation-transformation hypothesis, using in their experiments sandwiches of flattened presumptive epidermis, in which pieces of living notochord rudiment from a young neurula were used as inductor. At the periphery of the sandwiches only archencephalic differentiations were encountered, whereas in the central part of the sandwiches, closer to the inductor, differentiations of a more caudal type were formed.

In order to check the validity of Nieuwkoop's hypothesis, Johnen (1956), working under Nieuwkoop in the Hubrecht Laboratory, made further experiments in which she removed the implanted living notochord inductor from the epidermal sandwich after various intervals of time. She has shown that when the host epidermis was from *Triturus* and the implant was removed after 4 to 10 hours, the reaction, if any, was always purely archencephalic. If, however, the

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implant was removed later than 16 hours, there were also deuterencephalic differentiations to be found in the central part of the sandwich. The later the inductor was removed the more frequent the deuterencephalic formations became, until the differentiations of the sandwiches were like the controls, in which the inductors were not removed.

The interpretation offered by Nieuwkoop and his collaborators seems unavoidable, if the results of experiments with living inductor alone are taken into consideration. There are, however, certain results obtained with abnormal inductors which will not fit into Nieuwkoop's system, as we have earlier pointed out (Toivonen & Saxén, 1955 *a*, *b*). I could not rid myself of the idea that in the experiments of Nieuwkoop and his co-workers the sequence of the qualitatively different structures may depend largely on the permeability of the living material of the inductor, which regulates the diffusion of the active agents. In my opinion a more plausible explanation of their results would be as follows. The inductor used in these experiments contains two kinds of active principles, the neural and mesodermal inducing agents. The neuralizing agents diffuse more rapidly. They are thus able to determine alone, without mesodermalizing agents, the reactive material farthest from the inductor, whereas the reactive material closer to the inductor is under the influence of both the neuralizing and the mesodermalizing agents, which simultaneously determine its differentiation.

From my earlier experiments (Toivonen, 1953, 1954) I knew that alcohol-treated bone-marrow of the guinea-pig is an inductor of almost purely mesodermal differentiations. This fact seemed to offer a useful method of testing whether the mesodermal formations can occur without the preliminary 'activation', which, according to Nieuwkoop's hypothesis, would always be necessary for the 'transformation', i.e. before the mesodermal differentiations could be formed. In the spring of 1955, without knowing of Johnen's (1956) experiments, I hit on the idea of the same method as she used, but with alcohol-treated guinea-pig bone-marrow as inductor. I have given a condensed account of these experiments earlier (Toivonen & Saxén, 1955*b*, p. 23). The preliminary series was completed in the spring of 1956 with some additional experiments.

MATERIAL AND METHODS

The sandwich method was used. The host presumptive epidermis was from young gastrulae of the common newt (*Triturus vulgaris*). The inductor material was marrow from the thigh-bone of the guinea-pig. It was fixed in 70 per cent. alcohol for 24–72 hours, and washed in sterile saline for 1–2 hours before being used for experiments. The saline for culturing the explants was buffered with phosphate (Deuchar, 1953).

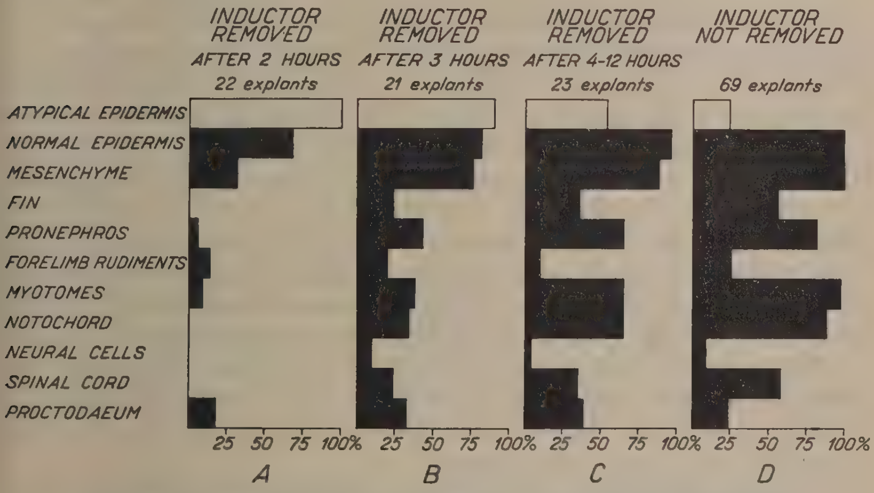
After 2, 3, 4, 5, 6, and 12 hours the explant was cautiously opened with a platinum needle and loop, the inductor tissue was removed, and the explant was cultured for about 2 weeks further. The removal of the inductor after 2 or 3 hours was very easy. After 4 hours and longer the inductor tissue was much infiltrated by living cells, and removal of the inductor was impossible without removing also a mass of living cells from the inner surface of the epidermis adhering to the inductor tissue. Microscopic examination of the slides proved, however, that removal of the entire inductor was always effected. The inductor was not removed from the control explants. They were likewise cultured for a further 2 weeks.

The explants were fixed in Bouin's fluid. They were first stained in bulk with borax-carmine, and the section counter-stained with picro-blue-black.

RESULTS

A detailed analysis of the structures induced in the different series of experiments is given in Text-fig. 1.

Series A. It will be seen that when the inductor was removed after 2 hours, at least a part of the explant always remained as undetermined atypical epidermis. In one-third of the cases no differentiation at all was found, as in the specimen shown in the Plate, fig. A. In the cases in which differentiations were visible, they were, in addition to the normal epidermis, very weak mesodermal ones, consisting almost solely of mesenchyme, as fig. B of the Plate shows. The induced proctodaeum seen in this case was also encountered in three others of this



TEXT-FIG. 1. Diagram showing the frequency of the various differentiations induced in presumptive epidermis explants of *Triturus*, when alcohol-treated bone-marrow of the guinea-pig was used as inductor. A–C, inductor removed after various intervals of time. D, control experiment, in which the inductor was not removed.

series. In my earlier experiments with bone-marrow I have interpreted the proctodaeum as the result of secondary induction by induced mesoderm (Toivonen, 1953). This explanation would also fit here.

I want to stress that after 2 hours' action of the inductor no neural differentiation was discernible, though the preparations were examined very carefully. Thus the first reaction of the epidermis does not appear to be a neural one, but purely mesodermal, if the inductor, like the bone-marrow used here, contains chiefly the mesodermalizing agents. Nevertheless, the action of the bone-marrow used for these experiments was not as purely mesodermalizing as in some of my earlier experiments (e.g. Toivonen, 1953), as the control series (Text-fig. 1) shows. In more than 50 per cent. of the cases a spinal cord was to be found. This was always very thin and short, however, and at the tip of the induced tail.

I also made some experiments in which the implant was removed after less than 2 hours. In these cases there was never any differentiation to be seen at all. Thus, 2 hours' action is to be regarded as the starting-point for any differentiation, when alcohol-treated guinea-pig bone-marrow is used as inductor in an epidermis explant of the newt embryo.

Series B. After 3 hours' action of the inductor all the differentiations to be found in the control series are present, although admittedly not as frequently. In most of the sandwiches (90 per cent.) the outer part of the explant, however, still remains undetermined atypical epidermis. In 25 per cent. of the cases there is now also a short spinal cord to be seen, as fig. D of the Plate shows. Mostly the reaction has remained weaker, as in the case illustrated in fig. C, in which, apart from the normal epidermis, only differentiation to mesenchyme is encountered.

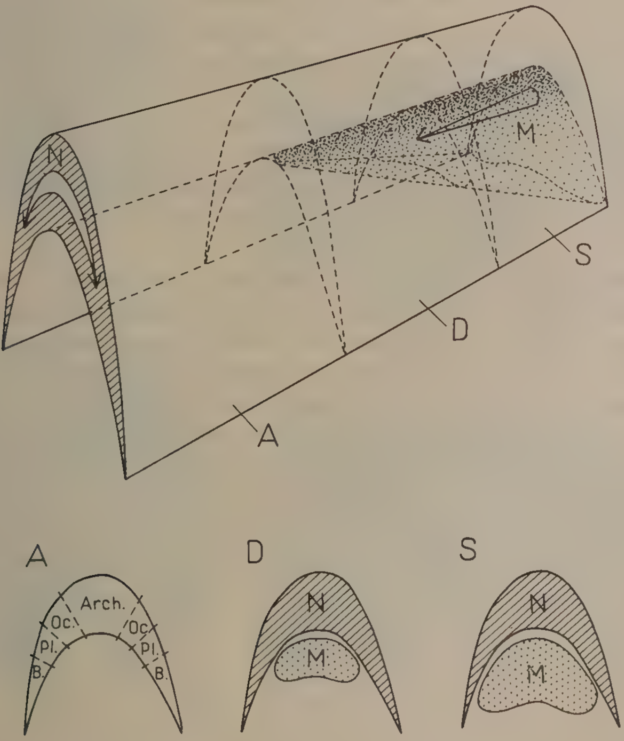
Series C. When the inductor is removed after 4–12 hours, the reaction of the epidermis is very like that of the controls. In half of the explants, however, there is still atypical epidermis, and all the kinds of differentiation are less frequent than in the controls. The lower frequency of differentiations compared with the control series may depend in part on the fact that the inductor tissue was always much infiltrated with all kinds of living cells, and, when the inductor was removed, some of the determined cells were also removed with it. For this reason I think it is correct to conclude that a time of about 4 hours would be long enough for the full action of the inductor.

Series D. In this series the inductor was not removed. In most cases (75 per cent.) the whole explant was differentiated to normal epidermis at least; in a quarter only, a part of the explant remained undetermined. Fig. E of the Plate shows a section of a specimen of this series with its differentiation. The spinal cord, which was encountered in half the cases, is the only neural formation.

DISCUSSION

According to the experiments reported above, it seems obvious that an inductor, which in its action is virtually purely mesodermalizing, will from the outset exert a mesodermalizing effect, without any preliminary archencephalic activation.

In the light of these experiments of mine, a more correct interpretation of Johnen's results would be that the living notochord, which she had used as inductor, contains both the active principles, the neuralizing and the mesodermalizing agents. The neuralizing ones diffuse more rapidly and can effect a labile determination. Afterwards, the stronger mesodermalizing agents are able to act simultaneously with the neuralizing principle and together they both determine the development of the region closest to the inductor into deuterencephalon and its derivatives, but the mesodermalizing action has no time to reach the periphery of the explant before it has lost its competence, and because of this the reaction there remains wholly archencephalic.



TEXT-FIG. 2. Scheme illustrating the gradients of the two inducing principles in normogenesis. N, neuralizing principle; M, mesodermalizing principle; A, archencephalic region; D, deuterencephalic region; S, spinocaudal region; *Arch.*, archencephalon; *Oc.*, eye; *Pl.*, placodes; B, balancer (Toivonen & Saxén, 1955*b*).

Finally, I should like to emphasize that both Johnen's experiments and these of mine reported here are wholly consistent with the hypothesis concerning the induction process presented earlier (Toivonen & Saxén, 1955*b*). In our opinion (Text-fig. 2), there are two principles in normogenesis, both of them forming gradients on the dorsal side of the embryo. The neuralizing principle (N) forms a dorsoventral gradient, a dorsal field with lateral decrements. This principle is responsible for the archencephalic differentiations, when it acts alone without the other principle, the mesodermalizing one (M). The mesodermalizing principle forms a caudocranial gradient. The weak mesodermalizing action with strong neuralizing action is responsible for the deuterencephalic differentiations. As the mesodermalizing action becomes stronger in the caudal part of the archenteron roof, it is there responsible, together with the neuralizing action, for the spinocaudal differentiations.

Both these active principles are connected with chemical agents, and they are chemically distinct (cf., for example, Toivonen & Saxén, 1955*b*).

This scheme of ours is very like the one proposed by Yamada (1950). Yamada, however, had some doubts about the chemical nature of the mesodermalizing principle.

As a matter of fact the difference between our hypothesis and Nieuwkoop's is largely terminological. Nieuwkoop's 'activation' process corresponds to the action of our neuralizing principle, and his 'transformation' process corresponds to the action of our mesodermalizing principle. But, according to Nieuwkoop, the very same factor, which is at first responsible for the unspecific 'activation' process, may afterwards cause the activated epidermis to be transformed, and he believes that transformation is only possible if the epidermis is preliminarily 'activated'. In contrast, in my opinion the two principles are different and they each have a more or less specific action. They can act alone and separately, but also simultaneously. The mesodermalization does not require preliminary activation; in experimental conditions it may also be direct, as in the experiments reported above.

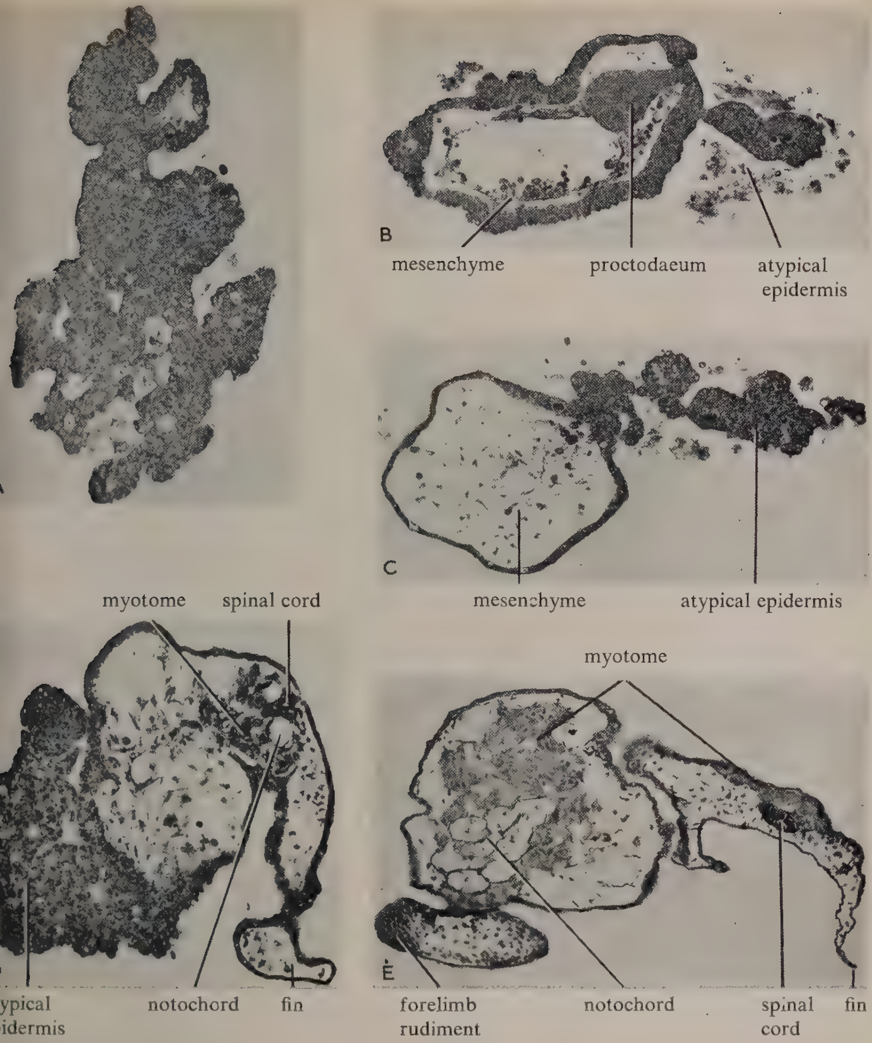
SUMMARY

1. Marrow from the thigh-bone of the guinea-pig, treated in 70 per cent. alcohol, has been used as inductor in experiments in which an epidermal explant from an embryo of *Triturus vulgaris* was used as reaction material.

2. The inductor was removed from the explant after 2, 3, and 4 to 12 hours. The explants were then cultured for about 2 weeks, as were the control explants from which the inductor was not removed.

3. The results of the experiments reveal that the first action of the inductor is a weak mesodermalizing, without any preliminary archencephalic activation such as Nieuwkoop *et al*. have postulated.

4. The validity of the hypothesis of Nieuwkoop *et al*. is discussed, and it is compared with that of Toivonen & Saxén.


B
mesenchyme
proctodaeum
atypical epidermis
C
mesenchyme
atypical epidermis
myotome
spinal cord
ypical
idermis
notochord
fin
myotome
E
forelimb rudiment
notochord
spinal cord
fin

S. TOIVONEN

ACKNOWLEDGEMENT

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EXPLANATION OF PLATE

FIG. A. Section through a presumptive epidermis explant from which the inductor was removed after 2 hours. Atypical epidermis only.

FIG. B. Section through a presumptive epidermis explant from which the inductor was removed after 2 hours. Besides normal epidermis, a weak mesodermal differentiation and a proctodaeum have been induced. Part of the explant remained undifferentiated.

FIG. C. Section through a presumptive epidermis explant from which the inductor was removed after 3 hours. Besides normal epidermis, only mesenchyme has been induced. Part of the explant remained undifferentiated.

FIG. D. Section through a presumptive epidermis explant from which the inductor was removed after 3 hours. Besides the normal epidermis, there are myotomes, notochord, a thin spinal cord, and a fin. Part remained undifferentiated.

FIG. E. Section through a control explant from which the inductor was not removed. The whole explant is differentiated.

(Manuscript received 18 : ii : 58)

Inversion of the Dorso-ventral Axis in Amphibian Embryos by Unilateral Restriction of Oxygen Supply

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INTRODUCTION

ACCORDING to the hypothesis advanced by Løvtrup (1958) the supply of oxygen is one of the factors responsible for the determination of bilateral symmetry in amphibian embryos. The protein coat covering the outside of the egg is known to have a very low permeability (Holtfreter, 1943), and it was suggested in the hypothesis that the formation of the grey crescent consists in a stretching of this coat by which the permeability is increased (cf. the work of Dalcq & Dollander (1948) and of Dollander & Melnotte (1952) on permeability of Nile blue). In this way the radial symmetry of the egg is changed to a bilateral symmetry from a metabolic point of view. As a consequence of the increase in permeability those oxidative, energy-supplying processes which are associated with gastrulation are enabled to proceed at a higher rate at one side of the egg. This hypothesis can of course only be valid for eggs which require oxygen for gastrulation. In some amphibian species, particularly in toads, gastrulation may occur under anaerobic conditions (Waddington, 1956). It seems necessary to assume that the bilateral symmetry is somehow built into the structure of these eggs.

In order to test the hypothesis mentioned one might cut off the supply of oxygen from the dorsal side of the egg, where the grey crescent is located, but not from the ventral side. This may be accomplished by placing the eggs in closed tubes with the grey crescent towards the closed end of the tube. Failure to develop would, of course, only permit the already-known conclusion that oxygen is necessary for gastrulation. Support for the suggested hypothesis might be found, however, if inversions of the dorso-ventral axis occurred, i.e. if gastrulation could be forced to take place at the ventral, aerobic side.

In the axolotl egg with which we have carried out our experiments it may be difficult to observe the grey crescent, and a different approach was therefore necessary. The first division plane does not invariably coincide with the future plane of bilateral symmetry (cf. the discussion by Ancel & Vintemberger, 1948).

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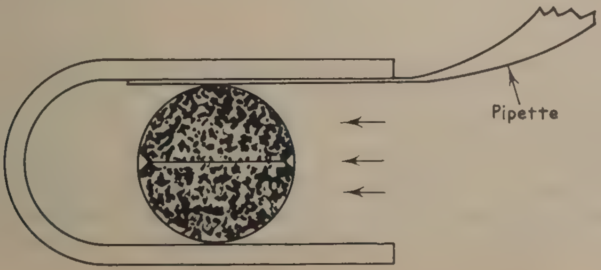
However, it is unquestionable that there will be an equal number of blastopores on each side of a plane going through the animal-vegetal axis perpendicular to the division plane, if the position of the grey crescent is either unknown or disregarded. In experiments with tubes as mentioned above, the possibilities are as follows. If oxygen were not essential for gastrulation, all eggs would gastrulate, about half of them towards the aerobic and the other half towards the anaerobic side. If oxygen is necessary two possibilities exist. If inversion is impossible, half of the eggs should gastrulate with blastopores towards the aerobic side, while the development of the other half should be blocked. Finally, if inversion were possible, significantly more than half of the eggs should gastrulate towards the aerobic side. This latter possibility, if it occurred, might be considered a support for the hypothesis advanced.

METHOD

Eggs of the axolotl (*Siredon mexicanum*) were used in our experiments. They are easily freed of the jelly coat and can be obtained relatively easily at different times of the year. The present experiments were carried out in May 1957 and January 1958. Glass tubes sealed at one end and about 3 mm. in length were prepared from Pyrex tubing. The internal diameter of the tube must be selected rather strictly, so that the eggs devoid of their jelly coats fit tightly into the tubes, without being appreciably deformed. The glass tubes were washed in hot chromic-sulphuric acid, and rinsed for 48 hours in running tap-water. They were then placed for a few days in daily changed distilled water. Eventually they were stored in distilled water. About 30–60 minutes before an experiment the distilled water was sucked out of the glass tubes, and they were subsequently transferred into Holtfreter solution.

A few hours after oviposition the eggs were transferred into dishes with Holtfreter solution. Eggs in the two-blastomere stage were picked out and freed of the jelly coat by means of watch-maker's forceps. These eggs were placed in the tubes with the division plane in the direction of the axis of the tube. As mentioned above, no attention was paid to the possible localization of the grey crescent. The eggs were sucked into the tubes by means of a very fine capillary pipette (see Text-fig.).



When the egg was properly placed and oriented within the tube, the pipette was withdrawn. The tubes with eggs were transferred to fresh Holtfreter solution, which was subsequently changed every day, and were kept at a temperature of 16°–17° C. After 24 hours the tubes were inspected. Eggs showing any signs of damage were discarded, and the same was done if the tubes were so wide that rotation of the egg was possible.

There were always some eggs which slipped out of the tubes during the first 24 hours of the experiment. These were used as controls, to determine the percentage of eggs which died before gastrulation. In the first experiments we tried to use as controls eggs placed in tubes open at both ends. However, it is difficult to place the eggs inside the tubes, as the very gentle 'sucking' procedure cannot be used, and when forced into the tubes the eggs slip out very easily.

When gastrulation had begun, after approximately 60 hours, the eggs were inspected every 12 hours.

RESULTS

The results of the experiments are summarized in the Table. The experimental eggs were divided into three groups: (1) gastrulation at the aerobic side; (2) gastrulation at the anaerobic side; (3) no clear gastrulation. These latter eggs died sooner or later. Control eggs were divided into two groups: (1) normal gastrulation; (2) no development.

TABLE

	Experimental eggs			*Control eggs*	
	Gastrulation at aerobic side	*Gastrulation at anaerobic side*	*No clear gastrulation*	*Normal gastrulation*	*No development*
May 1957 .	6	1	3	. .	. .
May 1957 .	37	. .	17	. .	. .
January 1958 .	111	7	8	21	2

The two preliminary experiments in May are not conclusive because of the lack of controls. The large number of eggs in the group with no clear gastrulation may be because development of the eggs is less satisfactory during the warm period of the year. A few observations were made on the gastrulation of the eggs in tubes, which deserve mentioning. First of all, whereas the beginning of gastrulation occurs within a rather narrow time interval in the controls, it was spread over about 2 days in the experimental eggs. In some of the 111 cases reported as showing gastrulation at the aerobic side, abnormalities in the gastrulation pattern were observed, indicating possibly the effect of inversion of the dorsoventral axis. Finally, we found that some of the eggs in the 'no clear gastrulation' group showed finger-like outgrowths, which means that whereas some processes were going on, gastrulation was completely inhibited.

DISCUSSION

The present experiments were performed in order to test the hypothesis of the mechanism of establishment of bilateral symmetry advanced previously. The significance of the results is unquestionable, and they give good support to the hypothesis. They do not, of course, give any definite proof; many more experiments will have to be performed before the validity of the hypothesis is established.

It may be argued that the inhibition of gastrulation at the anaerobic side is a result of an accumulation of metabolic waste products. This possibility certainly exists, but it does not explain the observed inversion of the dorso-ventral axis.

Another objection that might be raised is that the eggs, lying in the tubes, could perhaps rotate within their membranes. Against this it may be argued that the tubes in most cases were arranged so that the animal poles were turned upward; thus the driving force of rotation was absent. However, even if rotation occurred in a plane parallel to the axis of the tube, there should be the same probability that a dorsal side was changed from the anaerobic to the aerobic side, as that of the opposite event. Rotation in a plane perpendicular to the axis of the tube would be of no consequence. It should be mentioned though that the eggs fit very tightly in the tubes, so that rotation seems very unlikely to have occurred. However, in order to be completely sure on this point, some form of marking might be employed.

It should be mentioned that Lindahl a long time ago made some experiments with sea-urchin eggs which may indicate that similar conditions prevail in these embryos (Lindahl, 1936). Lindahl sucked eggs through fine capillaries, and the side of the egg which went first through the capillary almost invariably became the ventral side, i.e. the side at which invagination occurs. It is reasonable to assume that the membrane on the side of the egg going first through the pipette has been stretched most. If this is true, the increased permeability of the stretched membrane would explain the axial determination observed.

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Contribution to the Study of Germ-cells in the Anura

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WITH TWO PLATES

INTRODUCTION

THE problems of the germ-line of cells are of long standing in animal biology, but of these two surpass the rest in importance. At what stage in the life-cycle do the primordial germ-cells make their appearance, and do these gonocytes give rise to all, some, or none of the definitive sex-cells?

Since Nussbaum (1880, 1884) first discussed the continuity of the germ-cells from one generation to the next, study in this field of embryology has resulted in a measure of agreement that the primordial germ-cells make their appearance in the endoderm or mesoderm early in development.

The subject has been extensively reviewed in recent years by Bounoure (1939), Dantschakoff (1941), Everett (1945), Nieuwkoop (1946), and Johnston (1951). In the invertebrates, the origin of the gonocytes during cleavage is well established for some species (e.g. *Parascaris*), but, for the vertebrates, only Bounoure (1934) makes a claim for the formation of the primordial germ-cells as early as the blastula stage. His papers report germinal localizations in the cytoplasm of the fertilized egg of *Rana temporaria* which eventually become included in the primordial germ-cells. Apart from a report of a similar plasm in the early egg of *Xenopus laevis* by Nieuwkoop (1956 *a*, *b*), the claim of Bounoure occupies an isolated position, in spite of its obvious importance.

The work reported in this paper represents in the first instance a test of the validity of Bounoure's observations, but the author was interested also in examining the fertilized but uncleaved egg in greater detail, and in investigating stages earlier than those described by him. The extension of the cytological techniques involved to the eggs and embryos of other Anuran species seemed of importance in establishing the general status of Anuran germinal cytoplasm.

MATERIALS AND METHODS

The material employed in these investigations consisted of the eggs, embryos, and larvae of *R. temporaria*, *R. esculenta*, *Bufo bufo* and *X. laevis*. Eggs of the first three species were obtained from ponds in Surrey, from animals that had

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been kept in the laboratory for some time, and from pairs in amplexus brought into the laboratory to spawn. *Xenopus* eggs were obtained by the usual method of gonadotrophic hormone injection.

Most sections were cut at 5 and $7\frac{1}{2}$ μ and stained according to the procedure outlined by Bounoure (1934). Dehydration with butanol gave better results than ordinary laboratory ethanol, and it is most important not to post-chrome the material for longer than 50 hours at 45° C. if sections with reasonably intact yolk-endoderm are to be obtained. The adherence of yolk to the slide is facilitated by dipping the dewaxed sections in a weak solution of celloidin prior to staining.

Some sections were tested for their pentosenucleic acid (PNA) content using the method of Brachet (1940) as modified by Darlington & La Cour (1947).

RESULTS

The results are set out separately for each of the four species, but for purposes of comparison Table 1 shows the number of eggs and embryos (but not larvae) examined for each species, and the number positive for localizations of germinal cytoplasm.

TABLE 1

	Number of gonocytes examined, with number exhibiting germinal plasm in parentheses			
Description of stage	*R. temporaria*	*B. bufo*	*R. esculenta*	*X. laevis*
Unfertilized	7(—)	. .	. .	. .
Fertilized	35(29)	. .	. .	10(7)
Cleavage.	31(28)	13(12)	. .	19(16)
Blastulae	20(17)	7(7)	. .	16(12)
Gastrulae	25(22)	6(6)	12(—)	9(9)
Neurulae	21(17)	2(2)	13(—)	13(10)
Tailbud	7(4)	4(4)	. .	. .

In the case of *Bufo*, *Xenopus*, and *R. esculenta* the results are set out in a regular order of developmental stages, but not in the case of *R. temporaria*. For, since the blastula of this species is the most convenient stage for the clearest observation of germinal cytoplasm, it was thought best to deal with this first.

A. *R. temporaria*

1. *From the blastula stage to tadpoles of length 11–12 mm.*

The germinal cytoplasm of Bounoure may be found most easily in sections cut from early to mid blastulae where it is contained within cells sited between the floor of the blastocoel and the vegetal pole. It is stained a distinct purple and is always seen lying peripherally in the cells that contain it in an area free of yolk inclusions. The number of cells that possess the plasm varies between 5 and 7, each cell usually containing a single islet whose maximum area is about three times that of the nucleus. During mitosis, the germinal plasm usually, but not always, becomes confined to one only of the daughter-cells.

In late blastulae some cells may retain the plasm in the position as described above, but others clearly show the germ-plasm enveloping the nucleus as a 'cap'. A few cells may actually demonstrate the intracellular migration of the germinal cytoplasm from the cell periphery to the juxtanuclear position (Plate 1, fig. A). By the time gastrulation is really under way, every cell possessing germinal plasm exhibits it in the nuclear position.

During gastrulation the cells with germ-plasm, which now number between 11 and 23, are moved by the morphogenetic movements from a superficial position in the floor of the blastocoel to the region of the mid-endoderm of the young neurula. From this time forward, the plasm progressively loses its affinity for the stains employed until, by the time the larva is 12 mm. in length, the plasm no longer takes up stain.

No significant change in the number of plasm-bearing cells is to be observed during neurulation, and, indeed, no mitoses were ever seen in them during this period.

At hatching (larvae of about 8 mm.) the number remains the same, but in the intervening period the plasm-bearing cells have moved from their deep endodermal position, around the archenteron (which by this time has been reduced to a slit) on both sides, to the dorsal crest of the endoderm. Sections showing all stages of this migration have been obtained. The large and clear nucleus, with its two nucleoli and corona of deep-red germ-plasm, is clearly seen in each of the plasm-bearing cells of the endodermal crest (Plate 1, fig. B). The position of these cells is precisely the same as that described for the primordial germ-cells of other Anuran tadpoles at this stage by Kuschakewitsch (1910), Humphrey (1925), Cheng (1932), and others.

In the 10-mm. tadpole the cells with germ-plasm are to be found in a median ridge at the top of the dorsal mesentery; the outlines of the cells are more clearly seen if thionin or light green stains are used instead of methylene azur. The length of this ridge and the number of germ-cells in it for six larvae are given in Table 2.

TABLE 2

Larval length	*Germ-cell. no.*	*Length of ridge*
(mm.)		(μ)
9·4	31	550
10·0	47	600
10·2	41	480
10·0	33	700
10·0	. .	600
10·2	43	550

Stages demonstrating a 'migration' of the cells up the dorsal mesentery from the dorsal endodermal crest were never seen; as the endoderm becomes withdrawn from the region of the aorta, the ridge of cells becomes left behind during the formation of the mesentery. The median position adopted by the cells described was exactly the same as that described for the primordial germ-cells of this species at this stage by Bouin (1901), and there seems little doubt in consequence that the cells that contain germinal cytoplasm (now, however, only weakly staining) are indeed primordial germ-cells. Their cytological characteristics (such as the clarity of the nucleus, the possession of yolk platelets, &c.), apart from the germ-plasm itself, are consistent with this view.

In 11-mm. tadpoles the primordial germ-cells come to lie in paired gonadal rudiments, except anteriorly where the ridge is still median. At this time the germ-plasm no longer stains, but the abundance of juxtanuclear mitochondria, and the persistence of yolk platelets (which in other kinds of cell are almost always reduced in size and number, or, in the case of the somitic mesoderm and blood, used up) do not permit of any other interpretation but that the same cell-type is being dealt with. Between the primordial germ-cells, and also around them, lie cells with elongated, densely-staining nuclei. It was difficult to be sure that any of these peritoneal cells were not transforming into primordial germ-cells, but equally there was no indication that they were doing so. Germ-cell counts and ridge lengths for six tadpoles of 11–12 mm. overall length (snout to anus 4 mm.) are given in Table 3.

TABLE 3

Germ-cell counts for six 12-mm. tadpoles of R. temporaria. *The length between the snout and the beginning of the germ-ridge is about 2½ mm.*

Ridge length in μ	570	507	650	559	415	600
Germ-cell no. in left ridge	30	26	29	21	29	24
Germ-cell no. in right ridge	29	25	23	22	36	20
Germ-cell no. in median position	4	2	5	8	1	4
Total germ-cell number	63	53	57	51	66	48

The above observations confirm in almost every detail Bounoure's own findings, and show that certain cells of the blastula stage are directly ancestral to the primordial germ-cells, whether or not these latter cells actually give rise to definitive sex cells.

2. *From the blastula stage to the ovarian oocyte*

In cleavage stages, the islets of germ-plasm are found in the vegetal macromeres closely applied to the vertical cleavage cell-walls (see Plate 1, fig. E for an example from another species).

Now when batches of fertilized but uncleaved eggs came to be examined, islets of germinal cytoplasm could sometimes be found among the yolk platelets in the neighbourhood of the vegetal pole, sometimes not. This remained true when a fresh batch of eggs was prepared; in two samples totalling 12 eggs only 5 had positive examples of plasm. It seemed reasonable at the time to suppose that perhaps some of the eggs in the uncleaved samples were unfertilized, and, working on this assumption, material was prepared from a batch of eggs obtained by artificial fertilization.

The examination of sections of a sample of 5 eggs fixed immediately prior to the first cleavage (the first furrow was present only as a wrinkle at the animal pole) revealed islets of germinal cytoplasm lying in a region concentrated at the vegetal pole. Each islet measured about 10 μ and was surrounded by conspicuous mitochondria (see Plate 2, figs. A, D for examples of other species).

By contrast, sections cut from a sample of unfertilized eggs (fixed from the same batch later artificially fertilized) did not exhibit any germ-plasm. All 7 of these eggs were spherical in contrast to the oval shape of the eggs comprising the sample referred to in the previous paragraph, a point confirming the observation of Selman & Waddington (1955), that fertilized eggs become elongated in the animal–vegetal axis before the first cleavage.

Samples of fertilized eggs fixed 46 minutes, 1 hour 36 minutes, and 2 hours prior to cleavage all showed germ-plasm in sections, although the islets were smaller (7–5 μ) and more widely scattered over the periphery of the vegetal hemisphere the nearer fertilization the sample had been fixed. A sample of eggs fixed 15 minutes after fertilization did not exhibit any islets of germinal plasm in sections.

The most interesting sample studied had been fixed at 2 hours 26 minutes prior to cleavage (i.e. about 35 minutes after fertilization). The germ-plasm was seen in the process of formation in the vegetal cortical region of all 8 eggs in the sample. The cortex itself stains blue with the methylene azur, and the islets of plasm were seen as pale purple areas with mitochondrial associations occupying a very superficial position (Plate 1, fig. D). The sites of formation were widely spread over the vegetal hemisphere.

Sections of yolk-free oocytes from the winter ovary of a frog are found to be free of any localizations of cytoplasm that may be interpreted as germinal. The same is true of ovarian yolky oocytes.

3. *Parthenogenetic blastulae*

As the appearance of the islets of germinal cytoplasm is so closely associated with fertilization, it is of some interest to know whether this appearance is dependent or independent of sperm entry. Eggs were taken from a ripe female frog and stimulated to develop parthenogenetically by pricking with a tungsten needle to which were adherent traces of frog's blood, i.e. after the manner of Bataillon (1910 *a*, *b*; 1913).

A small sample of 6 normal-looking parthenogenetic blastulae showed the presence of germinal cytoplasm in cells below the blastocoel floor. Thus the appearance of germ-plasm in the fertilized egg seems to be independent of sperm entry and, in fact, is part of the cortical reaction that follows the fertilization process.

4. *Pentosenucleic acids (PNA) and germinal cytoplasm*

(*a*) *Ovary.* In the yolk-free oocytes the whole cytoplasm stains pink, and the nucleoli (of which up to 25 per section may be seen) a distinct red with methyl green-pyronin. In the yolky oocytes only the cytoplasm in the immediate vicinity of the nucleus stains pink, although red granules are also to be found in the cortical plasm.

When sections are treated with ribonuclease, the areas mentioned no longer stain pink. These results are in accord with those of Brachet (1940) and indicate the presence of PNA in nucleoli and cytoplasm.

(*b*) *Fertilized material.* Sections of 5 μ thickness were cut from a sample of 7 fertilized but uncleaved eggs fixed just before cleavage and mounted so that alternate sections appeared on each of two slides. For any one egg, one slide was treated with ribonuclease and then both slides were taken through the stain together. All untreated slides exhibit pink islets among the yolk platelets of the vegetal region, and these were indistinguishable in character from islets of germinal cytoplasm stained according to the methods of Bounoure. In treated slides, no part of the sections is stained pink and areas corresponding to those stained pink in the control slides are clear. Here, then, is an indication that germ-plasm is associated with a PNA component.

In blastula material, pink-staining areas were found in some of the endodermal cells. Unfortunately their outlines were indistinct and it was not possible to make an accurate estimate of the number of such areas. In ribonuclease-treated sections these areas are clear. An association of germ-plasm with PNA is again indicated, but not on such strong grounds as before.

When six gastrulae were sectioned the stain did not reveal pink areas in any cells of any section.

B. *B. bufo*

Islets of germinal plasm similar to those found in *R. temporaria* are to be found scattered among the yolk platelets in the vegetal pole region of eggs fixed prior to and during the first cleavage. At the 32-cell stage some of the vegetal macromeres show examples of germ-plasm in the position already seen in *R. temporaria*: however, the clarity of the plasm is very striking at this stage and the plasm is often surrounded by black pigment granules. When a cell adjacent to the developing blastocoel contains germinal plasm a narrow protuberance may sometimes be seen projecting from it into the lumen. This protuberance contains germ-plasm together with some yolk and marked pigment aggregations, and it seems reasonable to believe that some extrusion process, unknown for the eggs of *R. temporaria*, is under way.

In a sample of 7 mid-late blastulae germinal plasm was found with ease in sections cut from every one. The average number of plasm-bearing cells at this stage is 17. In 5 of the blastulae globules of germinal cytoplasm, yolk, and pigment were found lying free in the blastocoel (Plate 2, fig. B); the biggest of these were 65 μ in diameter. The areas of germ-plasm within the cells of the blastocoel floor measured about 35 μ in diameter.

Gastrulae and neurulae possess examples of juxtanuclear germinal plasm in cells lying deep in the endoderm. Here the plasm has lost its striking purple appearance and stains mauve; another feature is the density of the mitochondrial population around the nuclei of the plasm-bearing cells (Plate 2, fig. C). In four tail-bud stages examined, primordial germ-cells were seen between the mid-endoderm and the base of the archenteron. The fugitive staining of the germ-plasm made counting difficult in these embryos, but the numbers of 'positive' gonocytes in each were 11, 17, 17, and 14.

In 6 tadpoles of length 10–11 mm. the primordial germ-cells were found at the top of the dorsal mesentery and in the germ-ridges. The nuclei of these cells stain pale blue with the methylene azur, and are often bilobed. One or two red nucleoli may be present together with a circumnuclear cloud of mitochondria.

These results show that the history of the primordial germ-cells in *B. bufo* is essentially the same as that in *R. temporaria*, the main differences being the peculiar extrusion of germinal plasm into the blastocoel and the large mitochondrial populations in the germ-cells.

C. *R. esculenta*

Because of the difficulty experienced in trying to get freshly deposited spawn and the lateness of the season, only late stages of development were available for study. This was particularly unfortunate in view of the discovery that the blastula stage is the best stage in which to distinguish the germinal cytoplasm for the first time.

(*a*) *Tadpole stages.* Primordial germ-cells possessing the characteristics described by earlier workers in this field are easily found in sections cut from tadpoles of 12 mm. total length (Cambar & Marrott, stage 38). At this stage the cells are sited in the gonadal ridges in which the primary gonadal cavity has already made its appearance. The ridges are 350–450 μ in length and begin about 3 mm. posterior to the snout. The nuclei stain pale-red, are large and often possess two nucleoli, and are surrounded by mitochondria and yolk platelets (which by this time have disappeared from other tissues). The germ-cells are very numerous (Plate 2, fig. F).

Sections from all of 9 tadpoles fixed at Cambar & Marrott stage 30 demonstrated the process of the separation of the primordial germ-cells from the dorsal crest of the endoderm (Plate 2, fig. E), and in 7 tadpoles of stage 25 the gonocytes were seen in the dorsal crest of the endoderm. In these the average ridge length is 570 μ and the germ-cell number 26. The red corona, which is so characteristic of the gonocytes of *R. temporaria* at this stage (Plate 1, fig. B), was never seen.

(*b*) *Neurulae and gastrulae.* Of 13 neurulae examined no section showed any cell that could be confidently assigned to the germ-line. No germinal cytoplasm, as seen in *B. bufo* or *R. temporaria*, was to be seen, and the nuclear size and clarity varied widely from cell to cell.

The earliest stages examined consisted of 12 gastrulae at various stages of blastopore formation. No germinal cytoplasm was seen in any section cut from this material.

D. *X. laevis*

(*a*) *Cleavage.* A study of 29 eggs demonstrated the presence and similar position as in *R. temporaria* of islets of germinal plasm (Plate 2, fig. D).

(*b*) *Embryonic stages.* At stage 8 (*Xenopus* normal table, Nieuwkoop & Faber, 1956) the germ-plasm is confined to cells lying in the floor of the blastocoel, in which cells it consistently adopts a peripheral position. The areas are somewhat larger than in eggs of the common frog. Cases of the envelopment of the nucleus by the germ-plasm are found in stage 9.

In gastrula stages (11 and 12), the plasm is always investing the nuclei of cells containing it. From this time on the plasm assumes a slate-grey colour in sections stained with Altmann and methylene azur stains.

In neurulae the gonocytes are found deeply embedded in the endoderm, but they are sited much nearer each other than in *Bufo* or *Rana*. The germ-plasm is very conspicuous and occupies a large part of the cells that contain it; the yolk platelets in these cells are somewhat smaller than in cells that are not gonocytes.

(*c*) *Tadpole stages.* At the stage when the mouth is breaking through (stage 40), the primordial germ-cells are lying in the dorsal endoderm or to one side of the archenteric slit. The nuclei of these cells are large and associated with conspicuous mitochondria, but germinal cytoplasm fails to stain. Thus, by comparison with tadpoles of the common frog and toad, the primordial germ-cells of *Xenopus* are not so clearly demarcated.

In the latest tadpole stages examined (44–47), the gonocytes were either median or on each side of the dorsal mesentery in its upper attachment. Again, the appearance of these cells is not so impressive as in the other species, including *R. esculenta*, where the yolk platelets are large and numerous compared with those of the South African Clawed Toad.

DISCUSSION

The histological observations made on eggs and embryos of *R. temporaria* in this study substantiate Bounoure's descriptions of the germinal cytoplasm and its history in almost every respect. As this cytoplasm is the only specific germinal element of the cytoplasm reported for any vertebrate, as far as the author is aware, the simple confirmation of Bounoure's original findings is, perhaps, the most important outcome of this contribution.

Almost the same sequence of events leads up to the establishment of the primordial germ-cells in *X. laevis* and *B. bufo*, and it might be thought that the germ-plasm is a characteristic of all Anuran eggs were it not for our experience with *R. esculenta*. It is most unfortunate that these observations had to be carried out on such a small amount of material fixed later than when most of the interesting events in the history of the germ-cells in the other species were occurring. An examination of the early history of the primordial germ-cells in this species needs to be performed, although it may be that the germinal element in the cytoplasm of the gonocyte is not so localized as to be visually demonstrable.

The primordial germ-cells make their definitive appearance in late blastula stages, but cells of a germinal peculiarity extend back as far in development as the fertilized egg. Thus, while Nussbaum's theory of the continuity of the germ-cells remains still arguable, it appears no longer necessary to debate the origin of the gonocytes as being in the endoderm or mesoderm, but to view these cells as being specific elements whose history happens to coincide with the history of one or other of these germ-layers.

The appearance of the germinal cytoplasm shortly after fertilization, and its gradual loss of staining affinity following gastrulation, parallels the origin and disappearance of those 'Keimbahn determinants' that have been reported in certain invertebrates (see Hegner, 1914; Bounoure, 1939).

But have we any reason for believing the germ-plasm of the frog to be a germ-cell determinant? In the opinion of the author, if the germ-plasm is not an active determinant then it is closely associated with some invisible substance that is, in the same way that the determination of the germ-cells in *Parascaris* is effected by a difference in the cytoplasm near the vegetal pole (Boveri, 1910; Hogue, 1910). An incidental point to note is the polar position of the germ-plasm in the eggs of such invertebrates as nematodes and insects, as well as in frogs, shortly after fertilization.

From fertilization to the late blastula stage the plasm-bearing cells do not differ cytologically from other cells (with the exception, of course, of the germ-plasm itself). But when the plasm becomes juxtanuclear (as in Plate 1, fig. C), the cells that contain it become mitotically inhibited and retain their embryonic character (e.g. yolk platelets) while other cells are becoming increasingly specialized. While the early appearance of the germinal cytoplasm does not seem to have much significance therefore, the early establishment of the gonocytes might have a functional significance in 'protecting' the unspecialized nature of these cells from the influences that mediate determination and differentiation. While this is only a suggestion that does not receive much support from the work reported here, it is difficult to disregard the fact that the definitive gonocytes are formed just before gastrulation commences, that is, precisely before that period when so many other cell groups will be determined to develop in specific ways.

At this time also the loss of staining affinity of the germ-plasm, and the drastic reduction in its PNA content, point to important changes taking place within the gonocytes themselves.

Another striking feature of germinal cytoplasm is its constant association with large numbers of spherical mitochondria (Plate 1, fig. F). These become the more conspicuous as development proceeds, for the mitochondria of somatic cells become smaller and more difficult to see, except in the liver and intestinal wall. In neurula stages it is the mitochondrial population that first betrays the presence of gonocytes in sections (Plate 2, fig. C), and is thus of interest in that a similar characteristic has been reported for the primordial germ-cells of guinea-pig (Rubaschkin, 1910) and chick (Tschaschin, 1910).

At their first appearance the islets of germinal plasm are widely spread over the surface of the vegetal hemisphere. Their subsequent concentration at the vegetal pole and ascent of the cell-walls of early cleavage are almost certainly a visible manifestation of that polar ingression described by Schechtmann (1934). In other words, we are dealing with a passive migration. When the first four macromeres are formed, each contains more than one islet of plasm and it is probably a matter of chance, during the formation of the early blastula, how many islets come to lie in any one cell. The intracellular movement of the plasm in late blastula stages is by some unknown protoplasmic rearrangement. The displacement of the gonocytes from the superficial blastocoel floor to the deep endoderm by the morphogenetic movements of gastrulation may again be described as passive. On the other hand, the ascent of the gonocytes from their deep endodermal position to the germ-ridges cannot be ascribed to any known morphogenetic event, and this ascent is perhaps brought about by amoeboid movement of the gonocytes themselves. However, no preparation in this present investigation afforded any evidence on this latter point, and the author thus cannot agree with the observation of Bounoure that the primordial germ-cells do move in an amoeboid fashion.

The final migration of the primordial germ-cells from the dorsal crest of the endoderm to the germ-ridges is largely due to the withdrawal of endodermal material from the axial structures. This explains why 'strays' are sometimes discovered left behind in the gut-wall, an observation made in this study and by previous authors as well.

SUMMARY

1. Shortly after fertilization of the egg of *R. temporaria* there arise, near the vegetal pole, small islets of specifically stainable cytoplasm that eventually become included in cells lying in the blastocoel floor. From this time forward the special cytoplasm becomes increasingly difficult to identify with the cytological techniques used, but not until it is possible to confirm that the cells in question are primordial germ-cells. Thus the findings of Bounoure (1934) for this species are confirmed and extended.

2. This germinal cytoplasm has also been found in the eggs and embryos of *X. laevis* and *B. bufo*, but, rather surprisingly, not in *R. esculenta*.

3. The germ-plasm is, at first, rich in pentosenucleic acid (PNA) but loses this property shortly after an intracellular change in its position which occurs in the earliest stages of gastrulation.

4. Examination of parthenogenetically activated eggs reveals that germinal cytoplasm can be formed in the absence of sperm.

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Migrating germinal cytoplasm (dark bow) in cell of late blastula of *R. temporaria*. Note the copious mitochondria between the plasm and the oval nucleus. ×680.

FIG. B. Primordial germ-cell of *R. temporaria* at crest of endoderm. Note the large, pale nucleus containing two nucleoli (black dots), and the corona of germ-plasm (black ring). ×630.

FIG. C. Gonocyte in the blastocoel floor of a mid-gastrula of *R. temporaria*. Compare the pale nucleus and the dark ring of germ-plasm which surrounds it (at the bottom of the photo) with the smaller dense nucleus lacking plasm in the somatic cell shown at top of picture. ×400.

FIG. D. Formation of an islet of germinal cytoplasm (framed area) from cortical material, in *R. temporaria*. ×770.

FIG. E. Germ-plasm (dark area) lying apposed to cell-wall (the vertical line) of macromere of 32-cell stage in *X. laevis*. ×560.

FIG. F. The same islet as in fig. E but at magnification ×1,500. Note the granularity of the plasm and the absence of yolk from it.

PLATE 2

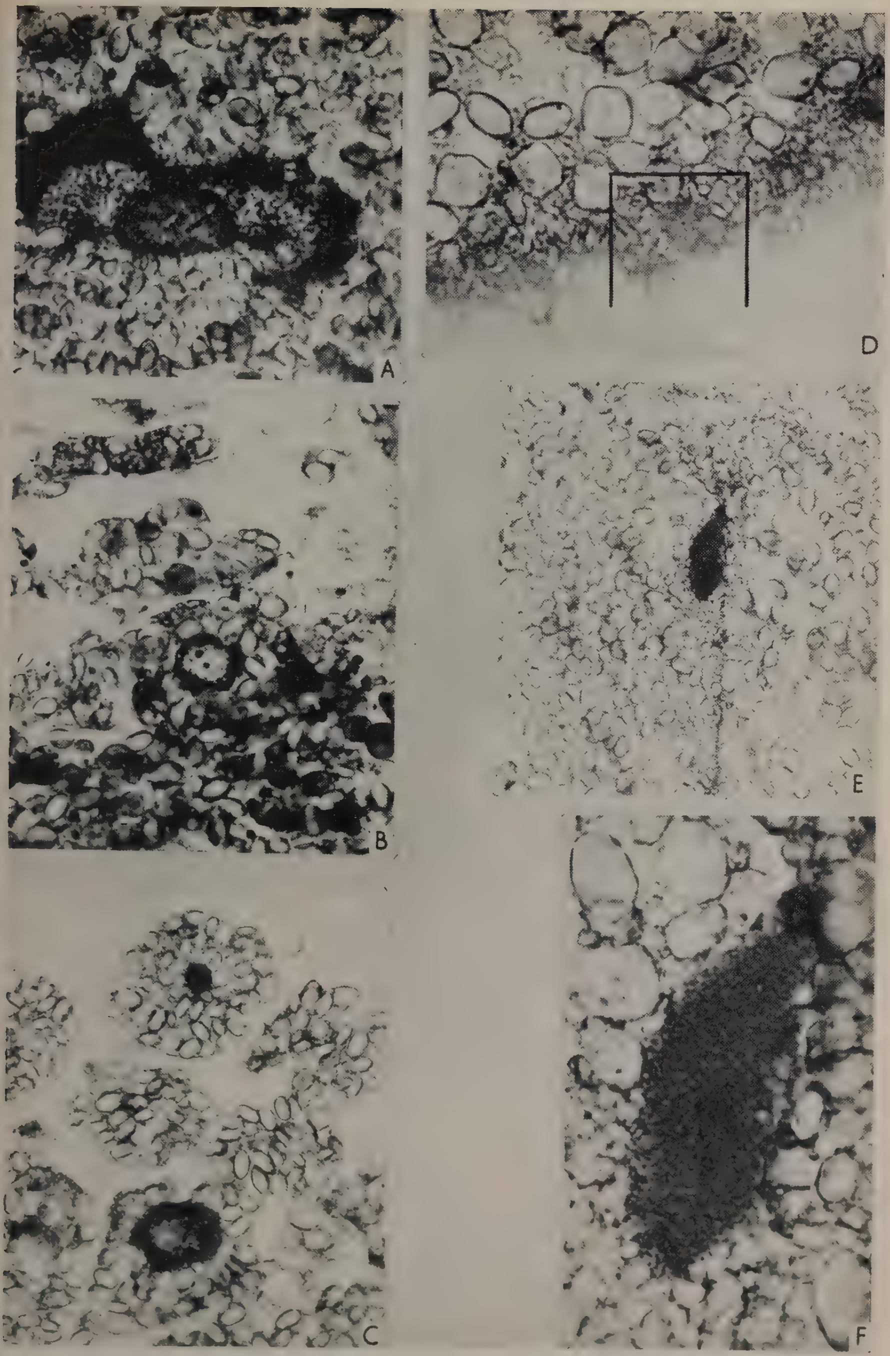
FIG. A. Two islets of germinal cytoplasm in the vegetal region of the fertilized egg of *B. bufo* The plasm areas are the two dark blotches. ×630.

FIG. B. Part of the blastocoel (the clear area) and blastocoel floor of *B. bufo* to show an isle of germ-plasm (at boundary of yolky material) and two globules of germ-plasm, pigment, and a little yolk lying free in the cavity. ×630.

FIG. C. Part of the floor of the blastocoel. containing two gonocytes (*gc*). from a young gastrula of *B. bufo*. Note the large numbers of mitochondria (small dots). ×630.

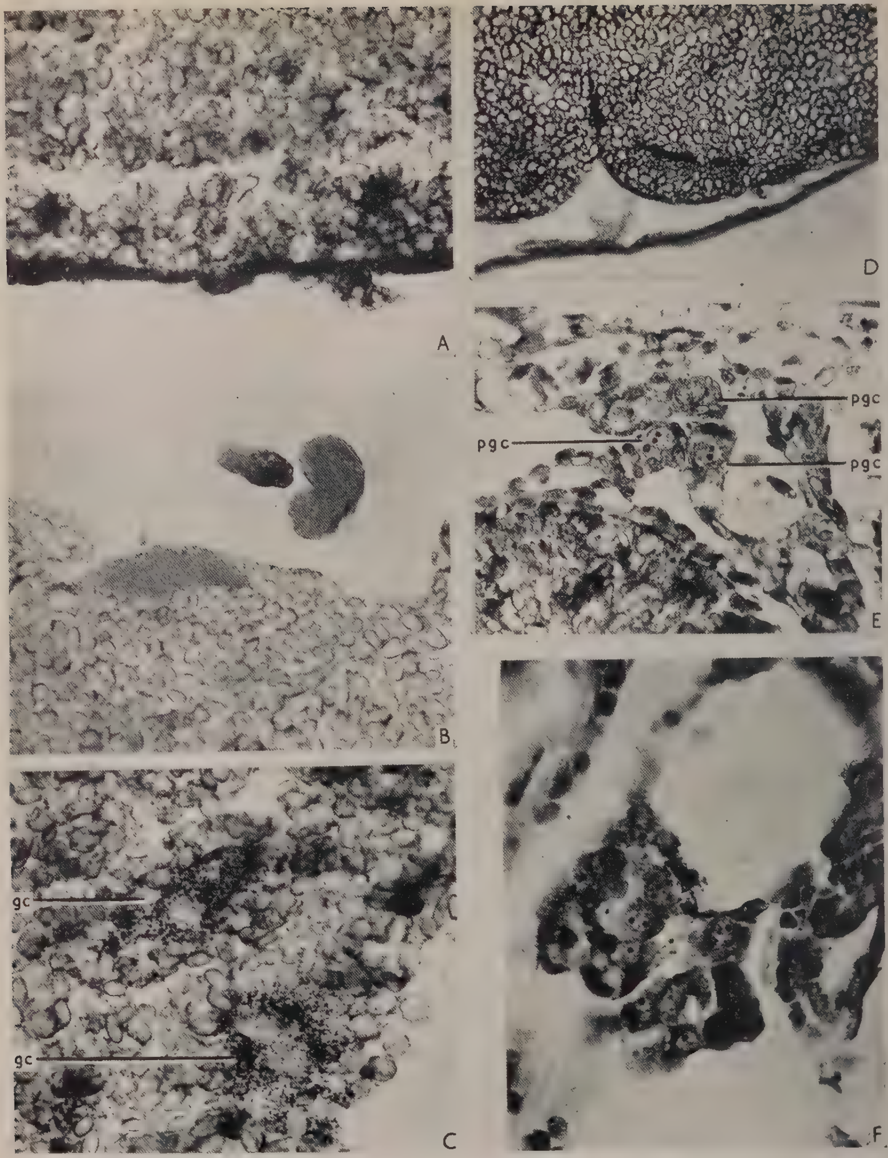
FIG. D. The extreme vegetal pole of a two-cell stage of *X. laevis* showing the islets of germ plasm (dark) among the yolk platelets. One islet is already adjacent to the first cleavage plane ×560.

FIG. E. Three primordial germ-cells (*pgc*) at the top of the dorsal mesentery in a tadpole of *R. esculenta* (the area with profuse yolk platelets is part of the endoderm, the oblique white area



A. W. BLACKLER

Plate 1



A. W. BLACKLER

Plate 2

is the coelom, and the germ-cells are lying at the end of the chain of cells—the dorsal mesentery—that crosses the coelom). Note the sharpness of the nuclear membrane and the presence of two nucleoli in two of the cells. The nuclei are pale compared with the nuclei of surrounding cells. ×450.

FIG. F. The germ ridges of a 12-mm. tadpole of *R. esculenta* with enclosed primordial germ-cells. Note the presence of two prominent nucleoli in the nuclei of some of the germ-cells. ×450.

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Acléiencéphalie expérimentale *in vitro*

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AVEC UNE PLANCHE

Le terme d'acléiencéphalie (du grec *κλειω* = je ferme) a été créé par Ancel (1955) pour désigner les monstres chez qui la gouttière nerveuse reste ouverte. C'est là une malformation qui peut être obtenue expérimentalement par des procédés divers: par *action des rayons X* (Kaven, 1938; Snell, 1941; Warkany & Schraffenberger, 1947; Russell, 1950; Hicks, 1953); par *action ultra-sons* (Lutz & Lutz-Ostertag, 1957); par *action de substances variées* (Ancel, 1955; Lefèvre-Boisselot, 1951; Giroud & Martinet, 1957); par *anoxie* (Gallera, 1951; Büchner, Rübsaamen, & Rothweil, 1951; Naujoks, 1953; Müshett, 1953; Büchner, 1955; Lutz & Lepy, 1958).

Une intéressante étude a été consacrée récemment à cette malformation par Giroud & Martinet (1957). Ils observent en particulier que le tube encéphalique ne se ferme pas au niveau du prosencéphale et du mésencéphale. Malgré cela une organogenèse presque normale s'effectue: des bulbes olfactifs s'y développent ainsi que des corps striés et des plexus choroïdes. Il se constitue ainsi un encéphale ouvert, de grande dimension. Ce n'est que secondairement que des processus de nécrose détruisent une grande partie de ces formations.

Dans des expériences antérieures nous avons déjà montré que l'acléiencéphalie peut être obtenue par action des ultra-sons sur le blastoderme non incubé. Dans ces expériences, le blastoderme est irradié à nu; l'œuf est alors refermé et ne sera ouvert, d'après la technique d'Et. Wolff, qu'après l'apparition de l'appareil circulatoire ($2\frac{1}{2}$ à 3 jours de développement). Il n'est donc pas possible d'assister à la mise en place de la malformation. Nous avons donc imaginé de reprendre ces expériences *in vitro*, à un stade où le blastoderme, d'après les données de nombreux auteurs, se cultive facilement, c'est-à-dire au stade de ligne primitive. Des expériences préliminaires *in vivo* nous avaient montré que des irradiations aux ultra-sons provoquent encore à ce stade, la malformation désirée.

Les cultures *in vitro* doivent ainsi nous permettre d'observer la formation de l'acléiencéphalie.

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MATÉRIEL ET TECHNIQUES

Les blastodermes au stade de ligne primitive ou de prolongement céphalique sont prélevés sur des œufs de Poule de race Gâtinaise. Les irradiations aux ultrasons sont faites à une fréquence de 1000 kHz et de 0,9 Wcm^2 de puissance pendant 5 minutes. Ils sont ensuite cultivés dans des salières sur milieu gélosé (milieu standard modifié de Wolff & Haffen, 1952).

Les embryons témoins sont cultivés de la même façon — mais sans subir d'irradiations aux ultra-sons.

RÉSULTATS EXPÉRIMENTAUX

Le développement des embryons témoins, non irradiés, est sensiblement normal (Planche, fig. 1), 30 embryons sur 31 sont normaux; un tel résultat peut être facilement obtenu à condition d'éviter toute dessiccation possible.

Par contre, les embryons irradiés présentent, dans 95 pour cent des cas (100 cas sur 106) la malformation recherchée.

Dès le stade 3 somites la plaque nerveuse, située en avant des somites, est plus étalée que normalement (fig. 2) et reste sur le même plan que l'épiderme voisin. Mais l'acléiencéphalie ne devient vraiment caractéristique et facilement reconnaissable qu'à partir du moment où le tube nerveux troncal se ferme (stade 7 à 8 somites) (fig. 3, 4, et 5). L'encéphale reste aplati; ses bords sont légèrement relevés du fait même que le soulèvement de l'embryon s'effectue normalement; les bords de la plaque nerveuse semblent alors être légèrement épaissis, alors que la région centrale laisse voir la chorde par transparence.

Or à un stade tel que celui-là, les bords de la plaque nerveuse devraient être entièrement relevés et soudés dorsalement; les principales vésicules de l'encéphale devraient être apparentes.

On remarque cependant que même le 'plateau céphalique' des acléiencéphales présente quelques différenciations; les régions antérieures, moyennes et postérieures sont séparées par de légères constrictions visibles macroscopiquement; de même, au niveau du diencéphale on entrevoit, dans certains cas, sur le bord du plateau, des invaginations correspondant aux évaginations latérales du diencéphale pour la mise en place des vésicules optiques primaires.

Ces caractères ne font que s'accentuer aux stades suivants; ainsi à un stade de 20 à 21 somites (fig. 6 et 7), les bords du plateau céphalique présentent des constrictions très nettes, divisant, dans tous les cas, le 'plateau céphalique' en 3 régions; l'ensemble ressemble à une feuille lobée. Sur les bords de ce plateau on observe un léger soulèvement; c'est à ce niveau que se place la séparation entre le matériel nerveux et le matériel ectoblastique. Ce soulèvement est plus marqué dans la région antérieure; les vésicules optiques sont, dans certains cas, visibles latéralement.

Enfin, vers le 3ème jour de culture (stade 30 à 40 somites), s'effectue la flexion caractéristique de la tête; dans la région antérieure l'ectoderme s'est fortement soulevé et recouvre partiellement la région antérieure du ‘plateau céphalique’; à ce niveau l’encéphale de l’embryon paraît alors être normal. Latéralement l’ectoderme effectue un soulèvement analogue, sans pourtant recouvrir le système nerveux.

La mise en place des vésicules auditives et dans certains cas, la formation des vésicules optiques (fig. 8 et 9) avec différenciation du cristallin est facile à suivre en étude histologique. Près du diencéphale, des épaississements de la lame nerveuse correspondent au début de la formation des corps striés.

Dans les expériences que nous signalons ici, nos observations ne portent que sur l’encéphale. Mais comme il s’agit de résultats obtenus à partir d’irradiations totales, des malformations peuvent aussi être relevées au niveau du système nerveux troncal (formation de spina bifida), du matériel somitique et de l’emplacement des bourgeons des ailes et des pattes.

DISCUSSION ET CONCLUSION

Le blastoderme de Poulet, pris au stade de la ligne primitive, se cultive bien *in vitro*; ce fait a déjà été signalé par Waddington (1932) et par Spratt (1948) qui ont employé différents milieux, à base de plasma sanguin, de jus d’embryon, de jaune d’œuf ou même des milieux synthétiques. Le développement de ces embryons est sensiblement normal.

Si ces embryons sont préalablement irradiés aux ultra-sons, on observe la formation d’embryons acléiencéphales. Il est ainsi possible de suivre heure par heure le développement de cette malformation. La non-fermeture de la gouttière nerveuse antérieure provoque la formation d’une sorte de ‘plateau céphalique’. Malgré cette grave perturbation, ce système tend à réaliser ses prestations normales: dans certains cas, l’évagination des vésicules optiques primaires apparaît ici sous forme d’invaginations qui peuvent cependant induire un cristallin; cette invagination s’effectue très latéralement, au contact même de l’ectoderme, si bien que l’œil pourra se former à son emplacement normal (fig. 8). Des constrictions apparaissent latéralement qui vont diviser le cerveau en ses principales régions; sur le ‘plancher’ on voit des différenciations — peu caractéristiques encore à ces stades — mais qui correspondent aux corps striés.

Giroud & Martinet (1957) ont parfaitement analysé ces formations chez l’embryon de Rat, sur des stades de développement plus avancés, et y ont reconnu des corps striés, des plexus choroïdes.

Ces monstres acléiencéphales peuvent donc subir au niveau de l’encéphale une organogenèse très poussée; Ancel (1955) signale déjà que l’encéphale ouvert des Oiseaux prend un développement normal.

La culture *in vitro* de cette malformation permet ainsi une analyse du développement de l’acléiencéphalie. Il n’y a pas réduction du matériel nerveux ou de ses possibilités comme le nom d’anencéphalie, généralement donné à cette malformation, pourrait le faire admettre; il y a seulement absence de ces mouvements morphogénétiques qui transforment la plaque nerveuse en un tube nerveux. Cela n'empêche pas l'organogenèse et l'histogenèse dans leur ensemble. Les différentes parties de la 'feuille lobée' qui se développe, correspondent aux hémisphères, aux lobes optiques et au cervelet. Enfin, il est intéressant de signaler la flexion de la tête; elle aura pour effet un léger recouvrement — mais secondaire — de la région antérieure de la plaque encéphalique; elle simule, à ce niveau, l'effet obtenu normalement par la fermeture de la plaque neurale. Cette flexion aura aussi pour effet le relèvement des bords latéraux de la plaque encéphalique — mais dans la région moyenne de cette plaque, elle ne pourra subvenir aux déficiences morphologiques antérieures.

RÉSUMÉ

Les auteurs décrivent dans ce travail les premiers stades du développement de l'acléiencéphalie; l'absence de mouvements morphogénétiques antérieurs, qui ferment la gouttière nerveuse, entraîne la formation d'une plaque encéphalique; celle-ci subit cependant des différenciations morphologiques et histologiques comparables à celles d'un embryon normal. Les phénomènes de flexion de la tête pourront entraîner une fermeture antérieure de cette plaque ainsi que le relèvement de ses bords latéraux.

SUMMARY

In this work the authors describe the early stages of development of acleiencephaly. The absence of the morphogenetic movements which normally close the neural tube leads to the formation of a plate-like brain which undergoes morphological and histological differentiation comparable to that of a normal embryo. The usual phenomena of flexure of the head can cause an anterior closure of this plate into a tube and also the elevation of its lateral borders.

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EXPLICATION DE LA PLANCHE

FIG. 1. Embryon de Poulet de 3 somites obtenu en culture *in vitro* à partir d'un blastoderme au stade de ligne primitive.

FIG. 2 à 7. Embryons de Poulet acléiencéphales à différents stades du développement, obtenus en culture *in vitro* après irradiation aux ultra-sons du blastoderme au stade de ligne primitive. L'embryon de la figure 2 présente une région antérieure (futur matériel céphalique) plus étalée qu'un embryon normal du même stade (fig. 1). Dans les figures 3 à 7 il y a déjà formation d'une véritable plaque céphalique lobée.

FIG. 8. Coupe transversale du 'plateau encéphalique' au niveau du diencéphale avec l'invagination formant les vésicules optiques primaires.

FIG. 9. Formation de cristallin chez un embryon acléiencéphale.

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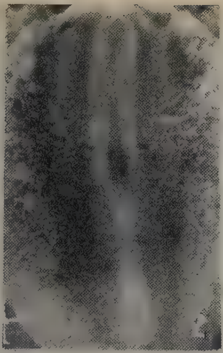

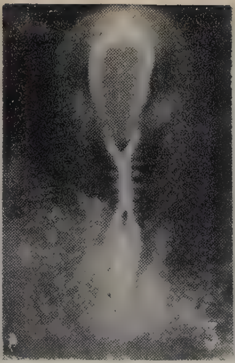

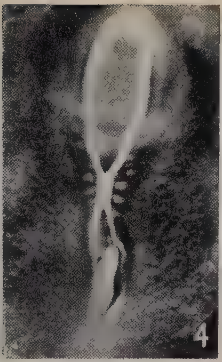

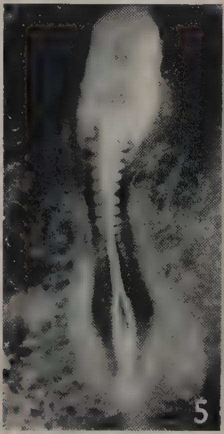

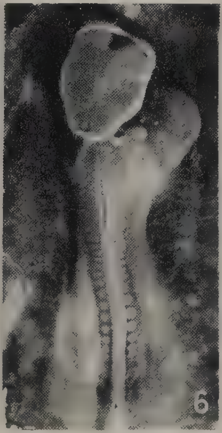

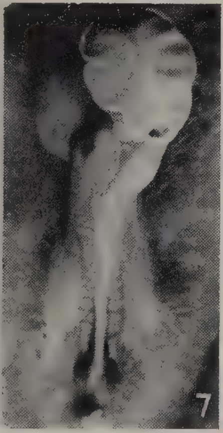

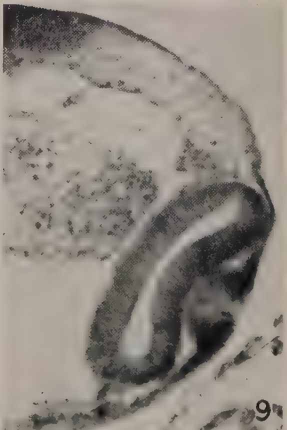

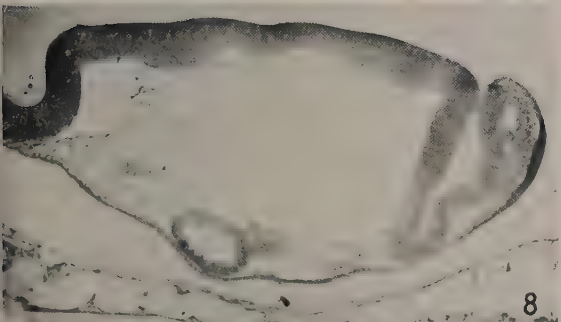

H. LUTZ *and* Y. LUTZ-OSTERTAG

Repair of Wounds of the Mucosa in the Rectum of the Cat

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WITH THREE PLATES

INTRODUCTION

INVESTIGATORS of wound healing in the alimentary canal have carried out their experiments mainly in the gastro-duodenal region, in view of the importance of the problem of peptic ulceration in man. Little attention has been paid to repair processes at lower levels of the intestinal tract; histological investigations on this subject in the large intestine appear to have been carried out only by O'Connor (1954, 1956) and by Lumb & Protheroe (1955). Sircus (1956) studied the ulceration that ensued in portions of colon that had been implanted into the stomach wall in dogs, but his interest lay in the mechanism of ulcer production rather than ulcer healing, and Truelove's (1957) biopsies from patients with ulcerative colitis were used largely to assess a method of treatment and not to investigate repair processes.

The present series of experiments was carried out in order to study the repair of mucosal lesions in the rectum of the cat. Routine histological studies were supplemented by histochemical observations on both the normal and regenerating tissues.

MATERIALS AND METHODS

All experiments were performed on healthy adult cats, a total of 34 animals being used. Under nembutal anaesthesia, a piece of mucous membrane 0·5–1·0 sq. cm. in size was removed *per anum* from the rectum, at a site on the dorsal wall 2 cm. proximal to the muco-cutaneous junction. The mucosa in this region is very mobile, a factor which allowed it to be picked up with forceps prior to cutting off the required area with scissors. The lesion so created can be conveniently referred to as an 'artificial ulcer'. The animals were allowed to survive after operation for periods ranging from 24 hours to 6 months.

Following death by coal-gas poisoning, the site of the lesion, together with an

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area of normal surrounding tissue, was removed and fixed in ice-cold 80 per cent. alcohol. Some specimens were fixed in Gendre's fluid after freeze-substitution. After embedding in paraffin wax, serial sections were cut at a thickness of 8 μ, and every twentieth mounted for routine staining with haematoxylin and eosin. These were examined, and from regions of particular interest serial sections were mounted for further study. The following techniques were used:

(1) Iron haematoxylin and picrofuchsin (van Gieson), for the demonstration of connective tissue.

(2) The Gomori cobalt sulphide technique for alkaline phosphatase, with the modification of Kabat & Furth (1941). Incubation times varying from a few minutes to 24 hours were used.

(3) The periodic acid-Schiff (PAS) technique for the demonstration of polysaccharides (Hotchkiss, 1948). Diastase-labile material, following digestion with saliva, was identified as glycogen.

RESULTS

Histological study of specimens from animals killed 24 hours after operation showed that at the ulcer site the whole thickness of the mucosa, including the muscularis mucosae, had been removed. The floor of the ulcer consisted of the inner layer of the muscularis externa, with some overlying submucosa and a variable amount of blood and fibrin clot. The epithelium and lamina propria at the ulcer margins had fallen towards the ulcer floor, thus covering up the cut ends of the muscularis mucosae, which may have assisted the process by retracting. In some specimens a few epithelial cells were found to be overlying the edge of the ulcer floor.

By the end of 2 days epithelial cells, in continuity with those of the undisturbed mucosa at the wound margins, were found covering the periphery of the ulcer (Plate 1, fig. A). Many of the cells were sufficiently flattened to give the appearance of a squamous epithelium (Plate 1, fig. E). During the next few days the spread or migration of epithelial cells towards the ulcer centre continued, but by the fourth day the very flat type of cell was no longer seen; all were becoming cuboidal or low columnar (Plate 1, fig. B). Among the many serial sections studied from ulcers in the first few days after operation, four sections only were found in which a single mitotic figure was seen among the spreading cells (Plate 1, fig. F). The apparent absence of goblet cells in the migrating epithelium was notable.

In the floor of the wound there was evidence of proliferative activity in connective tissue-cells from the second day onwards. This was not confined to regions near the surface of the wound; mitoses in connective tissue-cells were also found in the septa between bundles of muscle fibres in that part of the muscularis externa that lay below the ulcer area (Plate 1, fig. C). Beneath the spreading epithelium, and in unepithelialized areas in the centre of the ulcer, typical granulation tissue was appearing, as evidenced by the presence of young fibroblasts with well-marked cytoplasmic processes (Plate 1, fig. B), and buds of capillary blood-vessels.

There was considerable variation in the subsequent course of the healing process from one cat to another, particularly in regard to the amount of granulation tissue formed in the ulcer floor. Where the amount of such tissue was relatively small, ulcers examined at the end of one week after operation revealed that the epithelium had migrated for about 1,000 μ towards the centre of the lesion, and that in some places near the original ulcer margin it dipped down into the underlying granulation tissue, forming shallow epithelialized depressions (Plate 2, fig. 1). All the epithelial cells were now tall columnar in form (Plate 1, fig. G), and although some goblet cells were seen they were not numerous. Between the second and fourth weeks such lesions had become completely epithelialized, and showed over the entire wound area numerous shallow depressions (Plate 1, fig. D) whose epithelium contained occasional mitotic figures (Plate 1, fig. H) and some goblet cells (Plate 2, fig. J). These goblets were not as numerous as in normal crypt epithelium. The granulation tissue had become organized and mitotic figures in connective tissue-cells were almost absent.

In wounds that showed an exuberance of granulation tissue, shallow epithelialized depressions were not seen, and deep crypts, with many goblet cells, abutted against the mound of granulation tissue, the surface of which was not covered by epithelium (Plate 3, fig. O).

At the end of 3 and 6 months the whole ulcer area was covered with a mucosa that closely resembled the normal, but the muscularis mucosae was absent from the region of the wound and its cut edges remained to give some indication of the original margin of the lesion. Some specimens exhibited a rather shallow mucosa in which the crypts were a little less deep and less closely packed than normal (Plate 2, fig. K). Others showed strands of fibrous tissue that passed obliquely from the submucosa into the mucosa between groups of crypts (Plate 3, fig. P).

Histochemical reactions

The epithelium in the undisturbed mucosa gave a strong reaction for alkaline phosphatase at the striated borders of the cells (Plate 2, fig. L). The reaction could be seen with incubation periods of only a few minutes, and was present even in the deepest parts of the crypts, although it was less intense here than at more superficial levels. Specimens examined within the first few days after operation showed that the migrating epithelial cells gave a completely negative reaction for phosphatase (Plate 2, fig. L). At the end of the first week the reaction in the new epithelium overlying the ulcer floor was still negative, even in the cells that lined the shallow depressions near the ulcer margins (Plate 2, fig. M). However, by the end of the third week a reaction of normal intensity was seen in all epithelial cells (Plate 2, fig. N), and this degree of phosphatase activity was present at all subsequent stages of healing.

In the developing and maturing granulation tissue of the ulcer floor, there was a complete absence of phosphatase at all stages of the healing process examined, including those early stages at which proliferative activity of connective tissue-cells was observed (Plate 2, fig. M). Even after prolonged incubation periods of 24 hours this new tissue was still negative.

With the PAS technique, the epithelium in the normal rectum showed the presence of PAS-positive material in the position of the striated border (Plate 3, fig. Q). As with alkaline phosphatase, the reaction was also present in cells in the deepest parts of the crypts, though of diminished intensity. The material was not diastase-labile following digestion with saliva and hence was not glycogen, nor could the presence of glycogen be demonstrated in any other part of normal epithelial cells. The spreading epithelium that was seen in ulcers a few days old showed little or no PAS-positive material at the free borders of most of the cells (Plate 3, fig. R); the absence of PAS-positive material was not as complete as the absence of phosphatase. After 7 days a strong reaction had returned to the position of the striated border (Plate 3, fig. S), and as in the normal rectum the material resisted digestion with saliva. At the same time, a number of small granules, infranuclear in position and diastase-labile, were revealed in the tall columnar cells of this spreading epithelium (Plate 3, figs. T, U). Some of the cells lining the shallow depressions near the ulcer margin contained these glycogen granules (Plate 3, fig. V). It was not possible to demonstrate glycogen at any later stages, but the PAS-positive border was seen in all epithelial cells throughout the remainder of the healing process. Although a number of workers, e.g. Culling (1957) and Hale (1957), have recommended fixation in Gendre's fluid for the preservation of glycogen, the use of ice-cold 80 per cent. alcohol in the present circumstances appeared to be equally efficacious.

DISCUSSION

The results indicate that an effective restoration of mucous membrane occurs at the site of a mucosal lesion in the rectum, but the muscularis mucosae shows no evidence of restoration. The absence of regeneration in this component of the alimentary wall was noted during wound healing in the cat's oesophagus (McMinn & Johnson, 1958) and small intestine (McMinn & Mitchell, 1954), and has been repeatedly observed both in other animals and in man (Ivy, Grossman, & Bachrach, 1952). The migration of epithelium from normal mucosa at the ulcer margins and the accumulation of granulation tissue in the floor of the ulcer are phenomena that are typical of wound healing, but there are a number of features of interest that call for some discussion.

The problem of mucosal restoration

Concerning the problem of whether the mucosa is made good by the formation of new crypts, or by a rearrangement of old crypts as a result of contraction exerted by the fibrous tissue that develops in the floor of the lesion, it is pertinent to compare the results of the present work with the observations made by O'Connor (1956) on mucosal repair in the large intestine of the mouse. He concluded that the gap in the mucosa was filled by fibrous tissue that contracted and so approximated the cut edges of the mucosa. In the rectum of the mouse, the outgrowth of epithelium from the wound margins never exceeded 600 μ, and he found no evidence of new gland formation.

From the present work on the cat it could be suggested that the conditions seen in Plate 2, figs. O and P are comparable with the findings in the mouse. On the other hand, those in Plate 1, fig. D and Plate 2, figs. I–K might be cited as evidence of new crypt formation. It could still be argued that the shallow depressions seen for example in Plate 2, fig. J are merely old crypts that have been flattened and pulled towards the ulcer centre by the newly forming fibrous tissue. However, the relative absence of goblet cells, abundant in normal crypts, does not support this contention. Furthermore, the histochemical findings offer evidence that new crypt formation does occur, at least in some specimens, for the following reasons. It has been seen that during the first week 'new' epithelium, i.e. epithelium that grows out from the ulcer margins over the periphery of the floor, gives a negative reaction for alkaline phosphatase, in contrast to the epithelium of undisturbed mucosa that is always strongly positive. By the end of 7 days new epithelium still gives a negative reaction for phosphatase, but may now contain glycogen granules that are never present in undisturbed mucosa. Thus the absence of phosphatase and the presence of glycogen appear to be criteria of 'new' epithelial cells. Such criteria are present in cells lining shallow depressions near the wound margins, and since 'old' crypt epithelium always gives a positive reaction for phosphatase and never contains glycogen, the present histochemical findings support the concept of new crypt formation. It is not possible to assess the extent to which fibrous tissue contraction may be assisting the closure of the defect. The fact that after the end of 2–3 weeks the histochemical reactions of the epithelium had returned to normal renders these tests of no value in determining whether new crypt formation occurred at later stages.

It should be noted that in those specimens in which there are no shallow depressions near the ulcer margins, the granulation tissue is profuse and has reached the level of the tops of the normal crypts, or has even exceeded it, as in Plate 3, fig. O. Where such depressions are present and regarded as the precursors of new crypts, the granulation tissue is much less exuberant (Plate 2, fig. J). It seems possible that an end result, such as is illustrated in Plate 3, fig. P, where there are fibrous tissue strands between the crypts, may occur when granulation tissue is abundant and subsequently contracts; the shallower type of mucosa illustrated in Plate 2, fig. K may be a later development of the pattern of healing seen in Plate 2, fig. J, which with the histochemical evidence suggests new crypt formation. While either of these two postulated courses of events may occur, the factors that determine which of them takes place in any particular wound are unknown.

At higher levels of the alimentary tract it would appear that new gland formation is the rule during the repair of mucosal lesions. In ileal wounds in the cat McMinn & Mitchell (1954) noted the new formation of crypts and villi without any excessive fibrosis, while Florey & Harding (1935) found that new crypt and villous formation took place in healing duodenal lesions. In stomach wounds of the cat and other animals, a number of investigators have described the formation of new glands (Longmire, Beal, Lipmann, & Bishop, 1952; Williams, 1953; Myhre, 1956). Since O'Connor (1956) found no evidence of new crypt formation at any stage of healing in the rectum of the mouse, it would be interesting to know the nature of the healing process in the stomach and small intestine of this animal.

It should be noted that Lumb & Protheroe (1955), who studied repair following surgical trauma in the human rectum, considered that outgrowing epithelium was unlikely to form new crypts. The pattern of mucosal healing in the upper part of the human alimentary tract (e.g. stomach and duodenum) has led to the belief that new gland formation does occur (Ivy, Grossman, & Bachrach, 1952), findings that are similar to those in the experimental animal at comparable sites.

Histochemistry of the epithelium

The accumulation of glycogen granules, in epithelium wherein glycogen is not normally detectable histochemically, may be comparable with the increase that occurs in other regenerating epithelia, e.g. in the skin and oesophagus (Bradfield, 1951; McMinn & Johnson, 1958), although the amount seen in the rectal cells is small compared with that found in the other epithelia mentioned. The epithelium of the crypts of both small and large intestines normally displays a considerable amount of proliferative activity, but the newly formed cells that result from such mitoses never contain glycogen, so that mere youth of cells would not appear to be a factor concerned in glycogen accumulation. It seems more likely that a nutritional disturbance occurs due to an abnormal environment.

Mitoses in migrating epithelium

No mitotic counts were carried out in the present study, but there did not appear to be any grossly recognizable increase in mitotic activity in epithelium at the wound margins, whereas most other epithelia do show such an increase when regenerating. However, on the basis of studies with colchicine, it has been shown (McMinn & Mitchell, 1954; McMinn, 1954) that in the ileum there is no significant increase in mitotic activity at the margin of a healing wound, and that the presence of a gap in continuity does not serve as a stimulus to increased cell-division in the epithelium of that organ. It may be that the rectum is similar in this respect. The presence of mitotic figures in the migrating epithelium in the rectum is a phenomenon that has not previously been described in epithelium of the small or large intestines, but the finding during the present investigation of four mitoses in layers of spreading cells indicates that such cells are capable of dividing. Although the absence of proliferation in spreading cells has been held to be one of the characteristic features of wound healing in epithelia (Arey, 1936), the present authors have found many mitotic figures in the migrating cells of regenerating urinary bladder and gall-bladder epithelia (Johnson & McMinn, 1955; McMinn & Johnson, 1955, 1956, 1957). In the light of this evidence, some revision of existing concepts becomes necessary.

Alkaline phosphatase of granulation tissue

A number of workers (e.g. Fell & Danielli, 1943) have provided evidence suggesting that alkaline phosphatase is concerned with the elaboration of fibrillar protein. The complete absence of phosphatase in the maturing granulation tissue of rectal wounds in the cat does not support this hypothesis, but is in keeping with the findings of the present authors during wound healing in other hollow viscera in this animal. The matter is fully discussed elsewhere (Johnson & McMinn, 1958).

SUMMARY

1. Wound healing in the rectum of the cat has been studied following the creation of artificial ulcers by removing small areas of mucosa.

2. During the first few days of the repair process epithelial cells migrated from the wound margins over the floor of the ulcer, in which granulation tissue began to accumulate.

3. Mitotic figures were occasionally found in migrating epithelium.

4. The striated borders of normal epithelial cells gave a strong reaction for alkaline phosphatase and were PAS-positive. These reactions were not present during migration in the early stages of repair. Glycogen granules, not normally detectable histochemically in this epithelium, were found in migrating cells at the end of the first week.

5. The pattern of healing at later stages showed considerable variation, according to the amount of granulation tissue formed. Where the amount of such tissue was relatively small, increasing epithelialization occurred, with evidence of new crypt formation; where granulation tissue was exuberant, the margins of the original lesion may have been drawn together by the contraction of maturing connective tissue.

6. Alkaline phosphatase was absent from the developing and maturing connective tissue.

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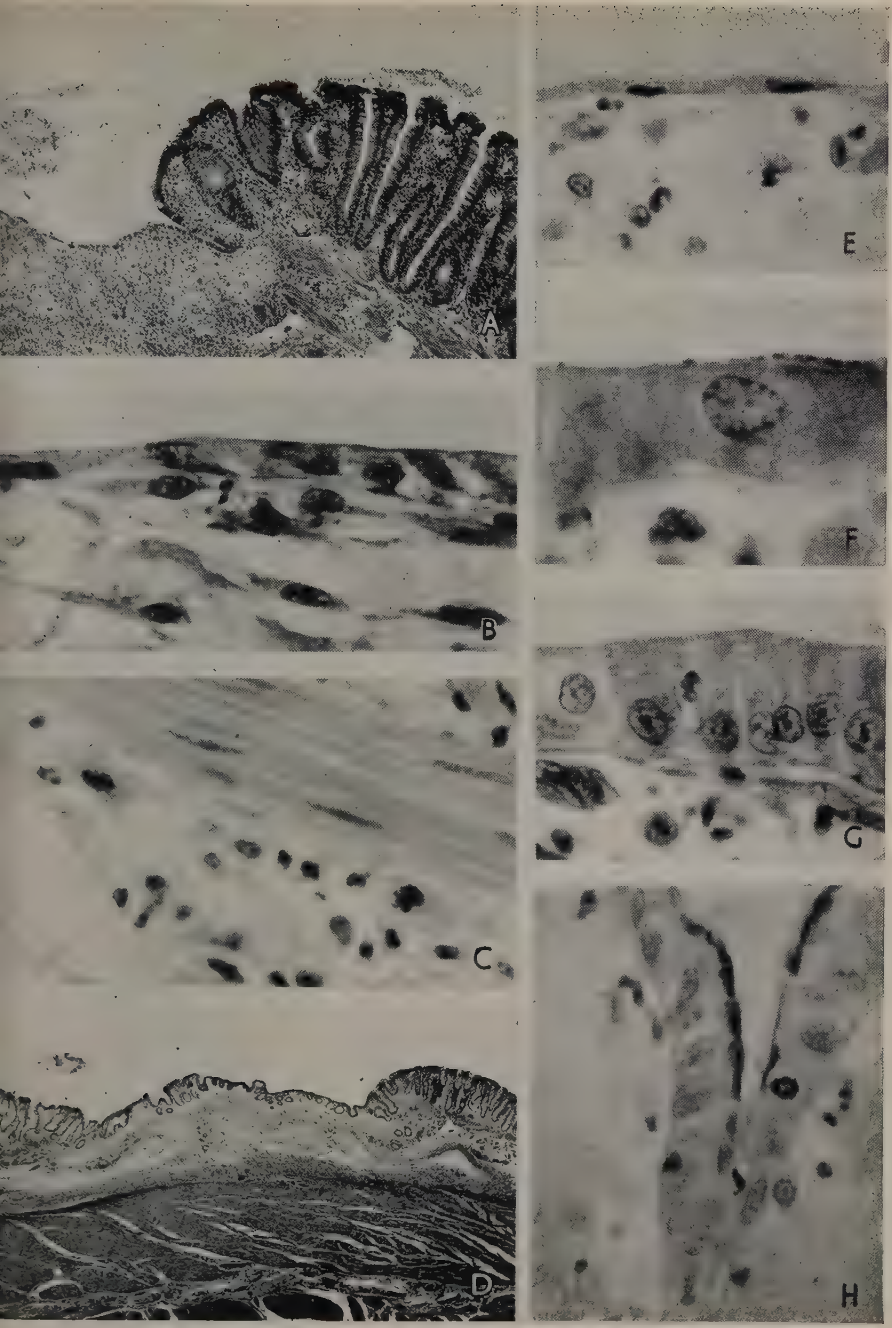
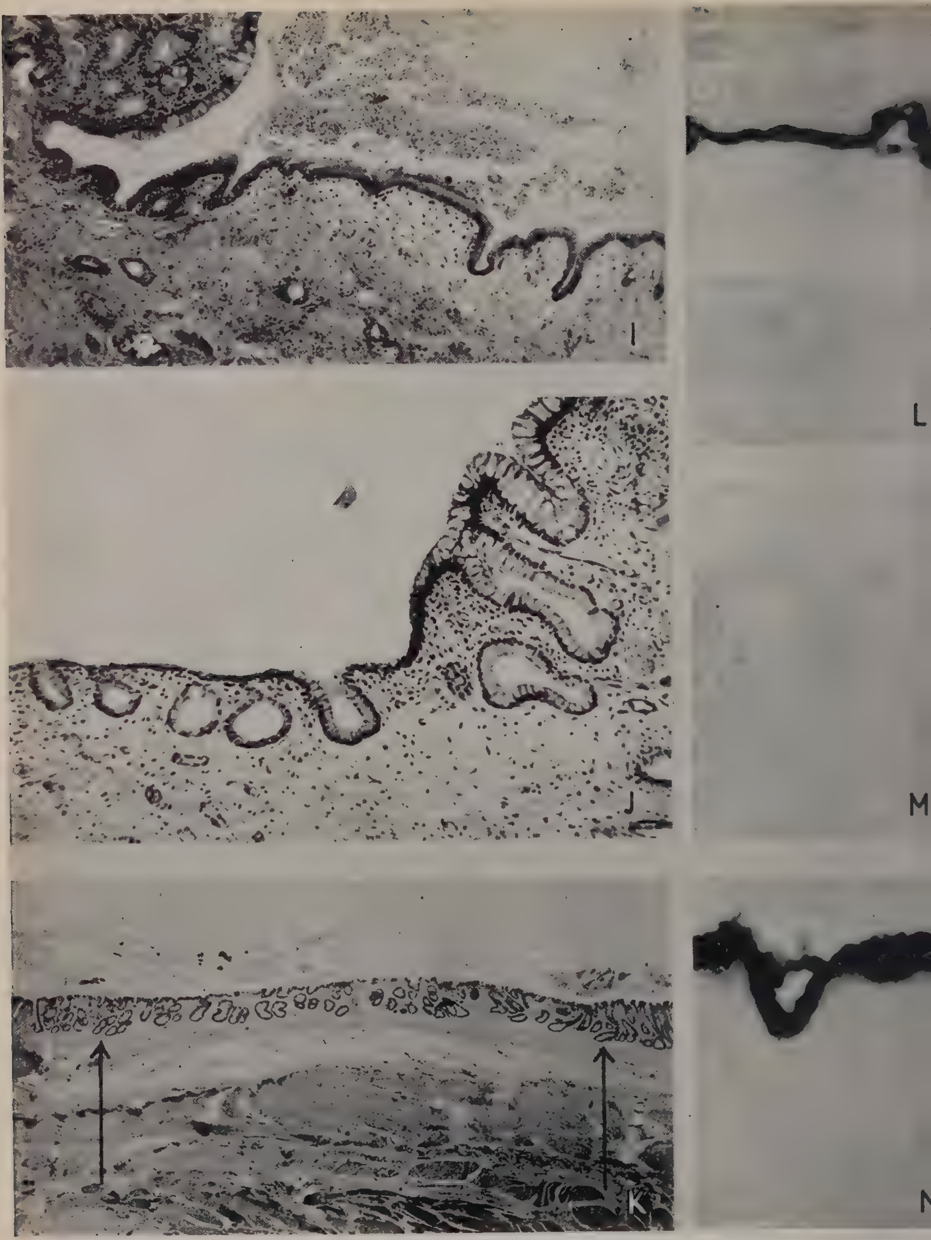


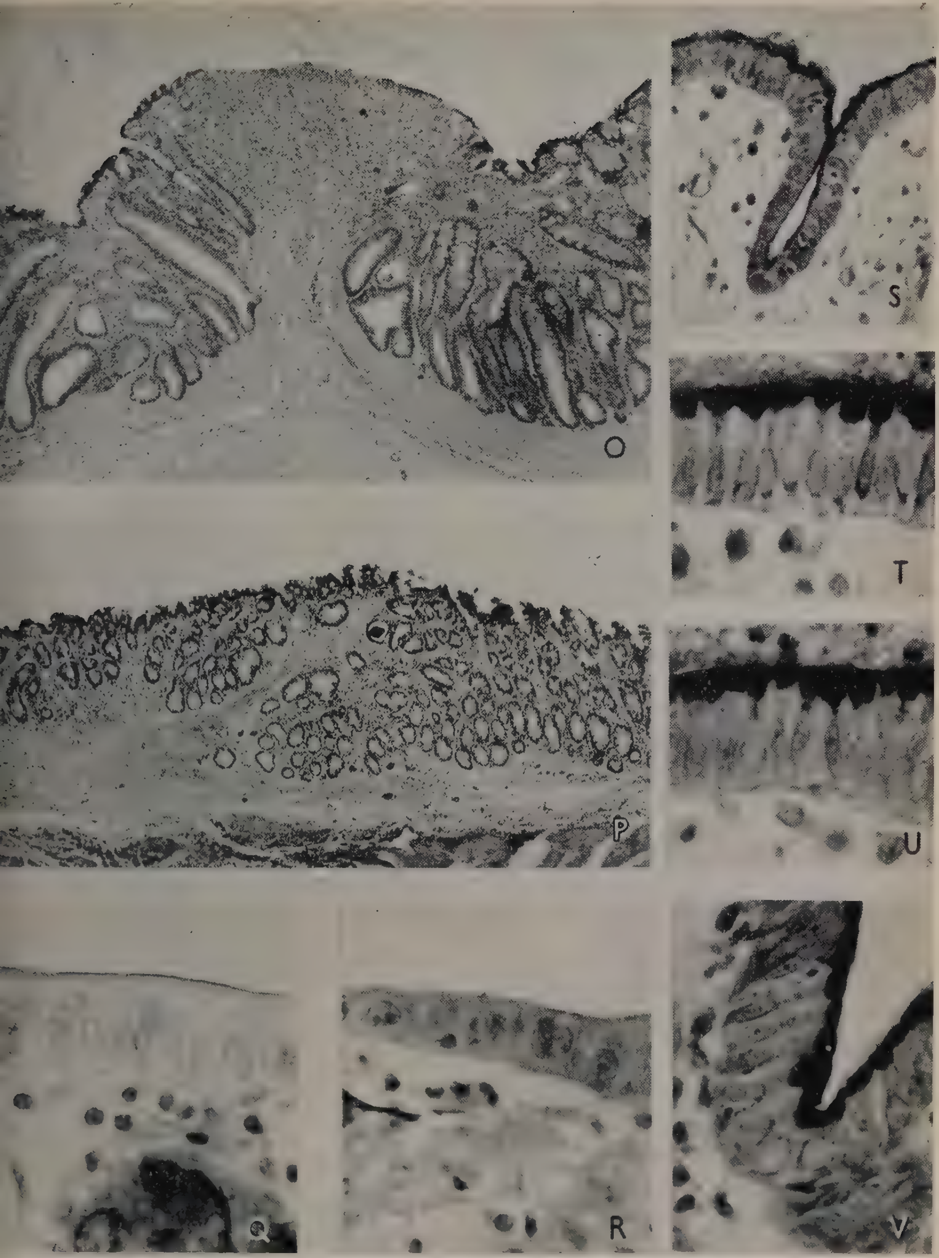
R. M. H. McMINN *and* F. R. JOHNSON

Plate 1



R. M. H. McMINN *and* F. R. JOHNSON

Plate 2



R. M. H. McMINN *and* F. R. JOHNSON

Plate 3

EXPLANATION OF PLATES

PLATE 1

FIG. A. Margin of an ulcer after 2 days. Epithelial cells are migrating over the ulcer floor. Note the cut edge of the muscularis mucosae. H. & E. ×65.

FIG. B. Epithelial cells overlying typical young fibroblasts of an ulcer floor, after 4 days. H. & E. ×640.

FIG. C. Two connective tissue cells in metaphase, lying deep to a bundle of muscle fibres of the muscularis externa below the floor of a wound, after 2 days. H. & E. ×640.

FIG. D. Ulcer site after 4 weeks, showing that the whole area is covered by shallow epithelialized depressions. H. & E. ×35.

FIG. E. Flattened epithelial cells migrating over the floor of an ulcer after 2 days. H. & E. ×640.

FIG. F. Migrating epithelium after 3 days, showing a nucleus in prophase. PAS. ×1340.

FIG. G. Migrating epithelial cells after 7 days. Compare with figs. B and E at the same magnification. H. & E. ×640.

FIG. H. A mitotic figure in the epithelium lining a shallow depression in an ulcer site after 2 weeks. PAS. ×640.

PLATE 2

FIG. I. Margin of an ulcer after 7 days, showing epithelial cells that have spread from the margin (on the left) dipping down into granulation tissue. Note that goblet cells are scanty in the new epithelium. H. & E. ×70.

FIG. J. An adjacent section to that illustrated in Plate 1, fig. D, at higher magnification. On the right is the mucosa of the original wound margin; on the left is the spreading epithelium lining shallow depressions and showing some goblet cells. H. & E. ×100.

FIG. K. An ulcer site after 6 months. The position of the cut edges of the muscularis mucosae is indicated. The area of the ulcer is occupied by crypts that are less deep and less closely packed than in the undisturbed mucosa. H. & E. ×15.

FIG. L. Above, normal rectal epithelium, showing a strong reaction for alkaline phosphatase at the striated border of the cells. Below, migrating epithelium after 3 days, giving no phosphatase reaction. Gomori technique, incubation time 30 minutes. ×640.

FIG. M. Epithelialized depression from an ulcer after 7 days, giving no reaction for phosphatase. Note that the granulation tissue is also negative. Gomori technique, incubation time 3 hours. ×255.

FIG. N. Epithelium from the centre of an ulcer after 3 weeks, giving a phosphatase reaction of normal intensity. The subepithelial tissue remains negative. Gomori technique, incubation time 30 minutes. ×640.

PLATE 3

FIG. O. Centre of an ulcer site after 4 weeks, showing exuberant granulation tissue that is not covered by epithelium. H. & E. ×50.

FIG. P. Ulcer site after 3 months. A strand or septum of fibrous tissue is seen passing from the submucosa between groups of crypts towards the surface. H. & E. ×35.

FIG. Q. Normal rectal epithelium, showing a PAS-positive border. PAS. ×640.

FIG. R. Migrating epithelium from an ulcer after 3 days, showing the absence of a PAS-positive border. PAS. ×640.

FIG. S. Epithelium from an ulcer after 7 days. A PAS-positive border has now returned. PAS. ×255.

FIG. T. Migrating epithelium from an ulcer after 7 days, showing infranuclear granules of glycogen. PAS. ×640.

FIG. U. An adjacent section to that illustrated in fig. T, showing that the material identified as glycogen is no longer present. PAS after saliva digestion. ×640.

FIG. V. Epithelial cells, with some perinuclear glycogen granules, lining a depression in an ulcer after 7 days. PAS. ×640.

(Manuscript received 27 : ii : 58)

The Behaviour *in vitro* of Dissociated Embryonic Pituitary Tissue

by H. SOBEL[1]

From the Strangeways Research Laboratory, Cambridge

WITH TWO PLATES

INTRODUCTION

It was previously reported (A. Moscona & H. Moscona, 1952) that the tissues of limb-buds and mesonephroi of early chick embryos can be dissociated into suspensions of discrete viable cells which, under certain conditions of cultivation *in vitro*, reaggregate into clusters and re-establish a tissue-like association. Upon further cultivation *in vitro* these primary cellular associations became transformed into organized tissue patterns, the development of which proceeds to the level of typical histological differentiation.

Owing to the nature of the experimental material studied so far, it has mainly been the capacity of the aggregates for re-establishing typical intercellular relationship that has come prominently into view. The present observations were aimed at examining the capacity of cells, aggregated from a discrete state, to resume and complete differentiation on the cellular level, e.g. to achieve a cytologically characteristic secretory status.

The normally developed cells of the anterior lobe of the pituitary carry a distinct mark of their state of differentiation—the secretory granules. Previous work has shown (H. Moscona & A. Moscona, 1952) that this degree of development can be achieved during cultivation *in vitro* by the isolated rudiment of the anterior lobe from the early chick embryo. An attempt was therefore made to dissociate the anterior lobe tissue into discrete, viable cells by enzymatic digestion of the intercellular cement and to study the capacity of such cells to aggregate and to differentiate *in vitro*.

MATERIAL AND METHODS

The experiments were made with anterior pituitary rudiments of chick embryos. The minute size of the rudiments made it preferable to take them at the latest possible stage, and it was found that the largest amount of still undifferentiated tissue could be obtained on the 8th day of incubation. At this stage the rudiment of the anterior lobe consists of a vesicle partially filled with elongated

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epithelial cords projecting from the walls (Plate 1, fig. A), and embedded in quite dense connective tissue. The intercellular substance of the connective tissue gives a positive McManus–Hotchkiss reaction, and the mucopolysaccharide material can be clearly demonstrated by this procedure as rather dense fibrils and granules. In some cells, granules that resist digestion with saliva are also revealed, and possibly represent the precursors of the future mucopolysaccharide material of the intercellular substance (Gersh & Catchpole, 1949).

The procedure of partial and complete disintegration of the pituitary tissue was based on that devised for limb-bud and mesonephric tissue of early chick embryos (Moscona, 1952).

The rudiments were dissected aseptically and after being rinsed in Tyrode solution were transferred to a saline lacking calcium and magnesium salts, in which they were incubated for 15 minutes at 38° C. The saline was then replaced by a 3 per cent. solution of commercial trypsin. During digestion the two parts of the pituitary could easily be separated owing to the loosening of the connective tissue between them; the posterior rudiment was rejected and the anterior cut into small pieces. The pH of the trypsin medium was gradually raised to 8·4–8·6 by the addition of 1 per cent. KOH solution. After about 15 minutes of incubation in this fluid the connective tissue was greatly hydrated and swollen, the epithelial tubules thus becoming clearly discernible as separate entities. The viscosity of the medium increased, presumably owing to the dissolved breakdown products of the intercellular substances. In contrast to the much younger tissues of the limb and the mesonephric rudiments, further disintegration of the pituitary tissue could not be achieved even after prolonged treatment with trypsin.

After exposure to trypsin, the pituitary tissue was therefore submitted to the action of *Clostridium welchii* collagenase.[1] The tissue was transferred to fresh saline and the enzyme, in powdered form, was added in a concentration of approximately 0·5 mg. of collagenase to 0·5 c.c. of fluid. After 10–15 minutes incubation in this solution at 38° C. the tissue broke down into a shapeless mass of loose mesenchymal cells and tubule fragments bound together by a viscous substance which was probably of a different nature from that released after trypsin treatment. At this stage the saline was replaced by several changes of Tyrode solution; care was taken not to disturb the main mass of cells but some, mainly from the connective tissue, were seen free in the fluid and were subsequently washed out. When brought in contact with Tyrode for a few minutes the partially disintegrated tissue regained a certain degree of compactness; in one series of experiments it was then explanted and cultivated by the watch-glass technique (Fell & Robison, 1929). The medium consisted of a clot composed of 2 parts of fowl plasma and 1 part of embryo extract, and the explants were transferred to fresh medium every 2 days.

[1] *Clostridium welchii* collagenase was kindly supplied by the Wellcome Research Laboratories through the courtesy of Mr. R. D. Thomson.

In other experiments incubation in the collagenase solution was prolonged for a further 5–10 minutes, when the epithelial cells of the tubules became loosened completely and were easily dispersed into a suspension by squirting them through a pipette of a suitable bore. They were then washed by centrifugation in the Ca- and Mg-free saline and Tyrode solution, and were finally resuspended for cultivation in 0·2 c.c. of a fluid culture medium in the cavity of a hollow-ground slide; the cavity was covered by a cover-slip sealed to the glass with molten paraffin-wax. Such a preparation was suitable for continuous observation of the cells.

The culture medium used was a fluid exudate from a fowl plasma-embryo extract clot. This was prepared by mixing plasma and embryo extract in equal quantities in a tube, crushing the ensuing clot and incubating it for 1 hour at 38° C. (H. B. Fell, personal communication). The decanted exudate was centrifuged to remove any extraneous cells.

The tissues were fixed in Zenker-formol for 1–3 hours. Sections were stained with Heidenhain's azan, Ehrlich's or Delafield's haematoxylin and eosin, or by the McManus–Hotchkiss periodic acid-leucofuchsin procedure.

Cell suspensions were fixed by placing a drop of the suspension on a cover-slip and exposing it for 10 minutes to acetic acid-formalin vapour and then floating the cover-slip face downwards on Zenker's fluid for 30 minutes. The cells were stained with either haematoxylin or azan and mounted on a microscopical slide.

THE BEHAVIOUR OF THE PARTIALLY DISINTEGRATED PITUITARY TISSUE

The development *in vitro* of the isolated pituitary rudiment, as described in detail in a previous paper (H. Moscona & A. Moscona, 1952) served as a standard of comparison for the behaviour in culture of the partially disintegrated pituitary tissue.

In a series of seven experiments, anterior pituitary rudiments of 8-day embryos were partially disintegrated by treatment with alkaline trypsin and subsequently by the action of collagenase as described in the section on techniques.

Histological examination of the partially disintegrated tissue fixed immediately after treatment revealed extensive structural changes. The epithelial cords were widely separated from each other by an optically structureless substance, in which small groups of cells and individual cells were embedded (Plate 1, fig. B). Most of the epithelial cords were broken into fragments and detached from the wall of the hypophyseal vesicle; the number of mesenchymal cells was greatly reduced. The appearance of the tissue indicated that the relationship between the mesenchymal and the epithelial components was profoundly disturbed in terms of their spatial arrangement as well as their relative proportions.

During the first few days of cultivation *in vitro*, the partially disintegrated tissue developed into typical cords and lobules, and became surrounded by a fibroblastic outgrowth similar to that of the untreated rudiments *in vitro*. Sections of explants fixed after 1 day in culture showed that the spaces between the cords and individual cells were considerably reduced. On the 2nd day the characteristic arrangement of cords and lobules was evident; they were packed closely together separated only by narrow strands of connective tissue (Plate 1, fig. C). During the following 2 days many mitoses were present. After 5 days *in vitro* typical acidophil and basophil cells had differentiated (Plate 1, fig. D). In the acidophil cells, most of the granules which stained orange with azan were formed on one side of the nucleus in the usual way, so that the nucleus became shifted to a terminal position. The basophils were of globular form with a slightly eccentric nucleus; minute dust-like blue granules were discernible in their protoplasm.

The formation of glandular cords and lobules and the appearance of chromophilic cells in the cultures of partially disintegrated pituitary tissue were comparable to similar processes in untreated pituitary rudiments cultivated *in vitro*.

THE BEHAVIOUR OF DISCRETE CELLS

Five to six rudiments were pooled for each experiment on complete dissociation of the anterior pituitary tissue. All suspensions were prepared by the method described in the section on technique. Two series of explants were made: (*a*) five cultures of discrete cells were grown for various lengths of time in the liquid culture medium; (*b*) eight cultures of cell aggregates were explanted on to the surface of a plasma-embryo extract clot and cultivated by the watch-glass technique.

Freshly isolated cells reacted by various changes in their form and behaviour to the conditions of isolation and the method of dissociation. In general these changes were similar to those described for the isolated chondrogenic and mesonephric cells (A. Moscona & H. Moscona, 1952). Two types of cells could be distinguished: relatively small cells, which protruded cytoplasmatic processes of various shapes and lengths; and larger cells, which tended to round up (Plate 1, fig. E). The formation of the protrusions might be interpreted as due to hydration of the cytoplasm resulting from the lytic action of the solutions used on the cell surface; similar reactions to lytic agents were observed in unfertilized sea-urchin eggs by Runnström & Monné (1945) and in isolated amphibian cells by Holtfreter (1948). The changes in the shape and behaviour of the cells were reversible as long as the nucleus was not affected.

When resuspended in the liquid culture medium the cells retracted their protrusions and rounded up. After about 30 minutes many of them began to move on the floor of the culture vessel, gliding by means of pseudopodia. Others accumulated in the centre of the concavity presumably by gravitation.

Series (a). After 12–24 hours in the liquid culture medium most of the discrete cells reaggregated into clusters of various sizes and shapes; the clusters were usually elongated, but a few had a globular form. During the next 24 hours the aggregates increased in length up to 0·1–0·2 mm.; the primary clusters appeared to unite in linear arrangement, the whole colony assuming a bead-like form. On more prolonged cultivation in the liquid medium these secondary aggregates did not develop and eventually degenerated.

Histological examination revealed that the primary clusters formed after 24 hours consisted of 3–4 globular masses loosely attached to one another (Plate 1, fig. F). Aggregates fixed after 48 hours in the liquid medium were composed of numerous round masses, some of them solid and others vesicular; mitoses were present. The whole structure was surrounded by a flattened cellular capsule (Plate 1, fig. G). The impression gained was that the colony consisted mainly of one type of cells, presumably epithelial.

Series (b). After 48 hours of incubation in the liquid medium the larger aggregates were explanted on the surface of a plasma-embryo extract clot for further cultivation. These explants behaved in a surprising way. They appeared transparent and healthy for a long time *in vitro*, but they did not develop to any appreciable extent. There was a slight liquefaction of the clot, but no fibroblastic outgrowth nor spreading of the tissue in the form of epithelial sheets. The cultures remained in a quiescent state, in which they could be maintained for more than 2 weeks, and eventually degenerated.

Sections of explants fixed after 1 day of cultivation showed clearly that the tissue consisted exclusively of epithelial cells. Mesenchymal elements were not included in the aggregates and did not form a peripheral outgrowth. A few mitotic figures were present at this stage, but after 3–4 days *in vitro* the mitotic activity subsided completely. In sections of cultures fixed after 14 days *in vitro* the cells appeared healthy, but they retained their rounded form and undifferentiated state (Plate 2, fig. H).

THE EFFECT OF ADDING CARTILAGE TO THE AGGREGATES

The substantial difference between the untreated pituitary explants and the pituitary cell aggregates was the absence of mesenchymal elements in the latter. A series of nine cultures was therefore made in which the epithelial aggregates were allowed to grow in contact with an extrinsic source of mesenchymal tissue. This was done by placing the aggregates in the immediate vicinity of an explanted fragment of cartilage from the phalanx of a 10-day embryo.

During the first 2 days' cultivation of the combined explants, a fairly dense fibroblastic outgrowth from the phalangeal perichondrium spread towards and partially surrounded the pituitary cells. The two tissues came into intimate contact so as to form a single unit, which could be easily handled during transfer or fixation. On the following day the fibroblasts completely surrounded the aggregates with a thin capsule, which thickened during further cultivation. An abundant outgrowth formed around the whole culture.

Sections of combined cultures fixed after 3 days *in vitro* showed that the fibroblastic tissue had encapsulated the aggregates completely and after 4 days had penetrated between the pituitary cells. The homogeneous epithelial tissue of the aggregates thus became subdivided into 'lobules' of epithelium surrounded by mesenchymal tissue derived from the fibroblastic outgrowth (Plate 2, fig. I). The pituitary cells looked healthy and mitotic activity was resumed. A few cyst-like structures filled with degenerating cells were found in the pituitary tissue. In sections of cultures fixed after 6 days *in vitro* chromophilic cells were seen to have differentiated (Plate 2, figs. J, K); they were predominantly acidophils and were situated mainly at the periphery of the lobules; basophils were very scanty.

Thus the addition of mesenchymal tissue to the aggregates of epithelial pituitary cells resulted in the transformation of the latter into a gland-like tissue, in which mitotic activity and cellular differentiation were resumed.

DISCUSSION

The method of dissociation of tissues into discrete cells, as devised for the limb and mesonephric tissues of early chick embryos (A. Moscona, 1952), when applied to the 8-day pituitary rudiment does not result in complete disintegration. This is probably due to the structurally more advanced and chemically more complex state of the connective tissue and of the cement substance of the pituitary rudiment at this stage.

On the assumption that at this age the intercellular fibrillar substance of the connective tissue might be of a collagenous or procollagenous nature, a procedure was evolved based on the combined action of trypsin and the collagenase of *C. welchii*; by this means the anterior lobe tissue was dissociated into discrete, viable cells. *C. welchii* collagenase was shown to dissolve native and degraded collagen (Oakley, Warrack, & Warren, 1948) and its action on the pituitary tissue supports the histochemical indications that collagenous material is present in the connective tissue of the gland at this stage.

The aggregates formed in the resulting cell suspensions consisted almost exclusively of epithelial cells. The reason for the disappearance of mesenchymal cells after the dissociation procedure was obscure at the time of the experiments. The recent observations of Shaffer (1956), however, showed that the α-toxin of *C. welchii* caused the selective destruction of mesenchymal cells *in vitro*. The concentration of the α-toxin present in the amount of collagenase used in our experiments was far above the effective minimum required for the differential destruction of mesenchyme. Thus the pure epithelial pituitary aggregates were obtained by the selective elimination of the connective tissue cells. It was interesting that these epithelial masses neither grew nor differentiated in the absence of connective tissue, but merely survived in a quiescent state.

The addition of connective tissue to the aggregates, however, caused them to resume their growth and development; first mitotic activity reappeared and ultimately the cells differentiated. These results imply an intimate histodynamic relationship between the two components of the glandular tissue. Flint (1903) and afterwards Moral (1915–16) suggested that a reciprocal morphogenetic action of the connective tissue and the epithelium exists in the developing submaxillary gland. Heidenhain (1921) in his 'synthetic theory of tissues' advanced the idea that the growing glands proliferate and develop as tissue systems, based on a close morphological and physiological relationship between all the constituents. Experiments *in vitro* with chick metanephros (Rienhoff, 1922) and mammalian submandibulary glands (Borghese, 1950) provided substantial evidence of the reliance of the epithelial anlage upon the presence of connective tissue in morphological development.

Although it can be assumed that in developing glands there exists an intimate relationship between the epithelial and connective tissues, there is at present no evidence as to the nature of their interactions. The complexity of the problem is stressed by the observations of Grobstein (1953 *a*, *b*, *c*), which suggest that the morphogenesis of some glandular epithelia, e.g. mouse submandibular gland, depends upon the specific properties in the mesenchyme normally associated with them. On the other hand, the present experiments with chick pituitary *in vitro* showed that the typical differentiation of the glandular epithelium can be achieved in association with extrinsic homologous mesenchymal tissue.

These results are corroborated by the more recent experiments of Grobstein (1955) in which it has been shown that the capacity of inducing and promoting the differentiation *in vitro* of some isolated glandular epithelia, e.g. nephric tubules, is not limited to the original mesenchyme of the glandular rudiment.

SUMMARY

1. A method for the partial or complete disintegration of the pituitary tissue of the 8-day embryonic chick is described.

2. The anterior lobe, after being partially disintegrated by incomplete dissolution of the intercellular substance, developed *in vitro* into typical glandular tissue.

3. After dissociation of the anterior lobe into a suspension of discrete cells, the epithelial cells reaggregated under suitable conditions *in vitro*.

4. The epithelial aggregate remained in a quiescent but apparently healthy state during further cultivation.

5. The addition of perichondrial connective tissue to the aggregates caused them to resume growth and development and to differentiate into typical anterior lobe tissue.

6. The mutual relationship of the connective and epithelial tissues in developing glands is discussed.

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Longitudinal section through the anterior lobe of the pituitary of an 8-day chick embryo, showing the hypophyseal lumen (HL) and the developing cords. Ehrlich's haematoxylin, eosin. ×480.

FIG. B. Section through the anterior lobe after partial disintegration by treatment with solutions of trypsin and collagenase. Azan. ×240.

FIG. C. Section through a culture of partially disintegrated anterior lobe tissue after 2 days *in vitro* showing formation of cords and lobules. Ehrlich's haematoxylin, eosin. × 160.

FIG. D. Section through a culture of partially disintegrated anterior lobe tissue after 5 days *in vitro* showing differentiated acidophil (A) and basophil (B) cells. Azan. × 1,140.

FIG. E. Smear of a suspension of pituitary cells in the treatment medium. Many cells show pseudopodial protrusions (P); others are rounded (R). Delafield's haematoxylin, eosin. × 960.

FIG. F. Section through a primary aggregate formed during 24 hours in a suspension of anterior lobe cells in liquid culture medium. Azan. × 240.

FIG. G. Section through a group of clusters formed in a suspension after 48 hours of cultivation in liquid medium. Azan. × 240.

PLATE 2

FIG. H. Section through a group of aggregates cultivated on a plasma-embryo extract clot for 14 days. Note the healthy appearance of the majority of cells. Azan. × 720.

FIG. I. Section through a culture of aggregates grown alongside a piece of cartilage for 4 days, showing penetration of fibroblastic strands between the epithelial cells and formation of small lobules. Azan. × 480.

FIG. J. Section through a culture of aggregates combined with cartilage, after 6 days *in vitro*, showing a glandular lobe in which mitoses (M) and chromophilic cells (CH) appear. Azan. × 960.

FIG. K. Another section through the same culture as above, showing mitoses and acidophil cells under higher magnification; A, acidophil cells; M, mitosis; C, cartilage; PT, pituitary tissue. Azan. × 1,140.

(Manuscript received 6 : iii : 58)

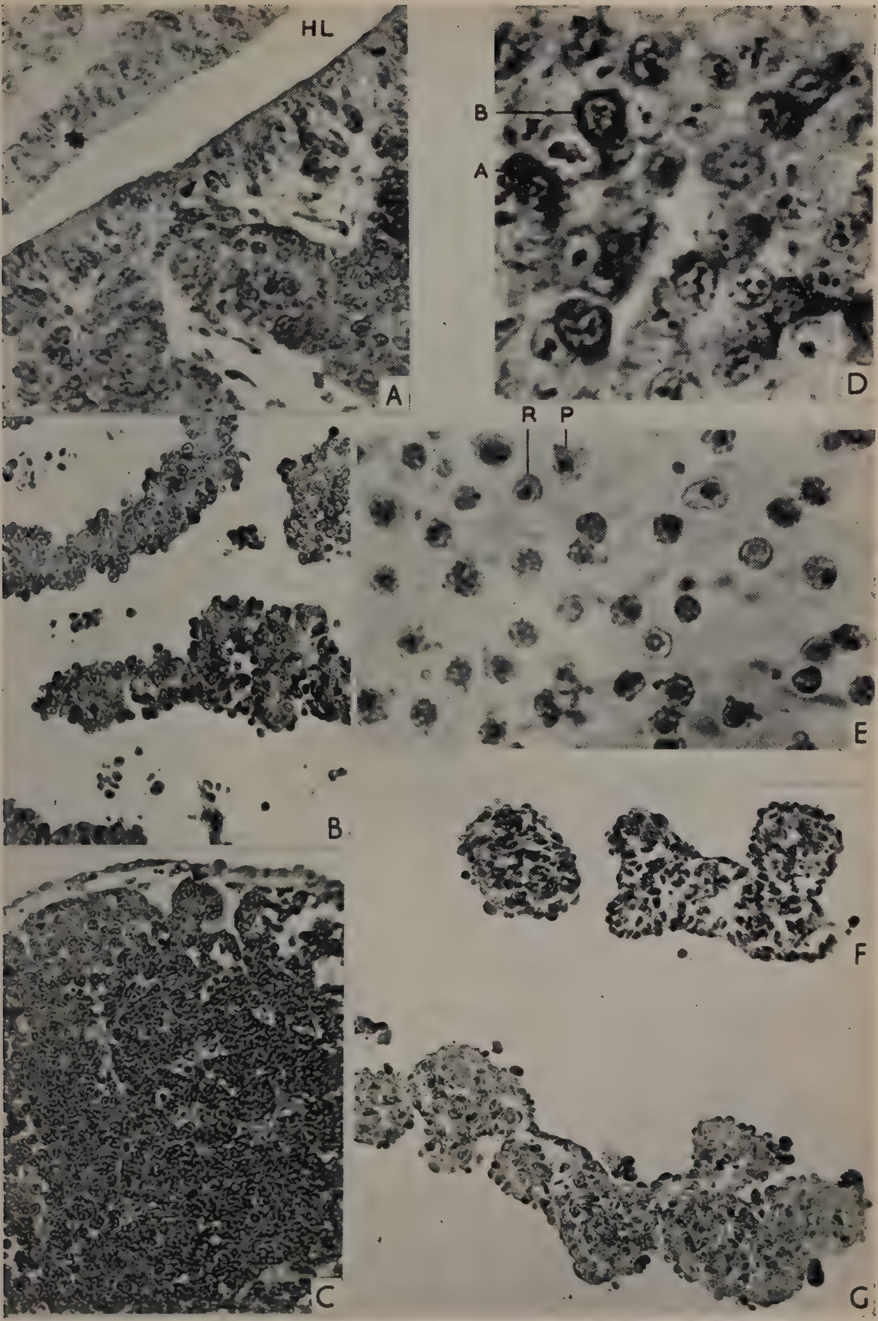

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Plate 1

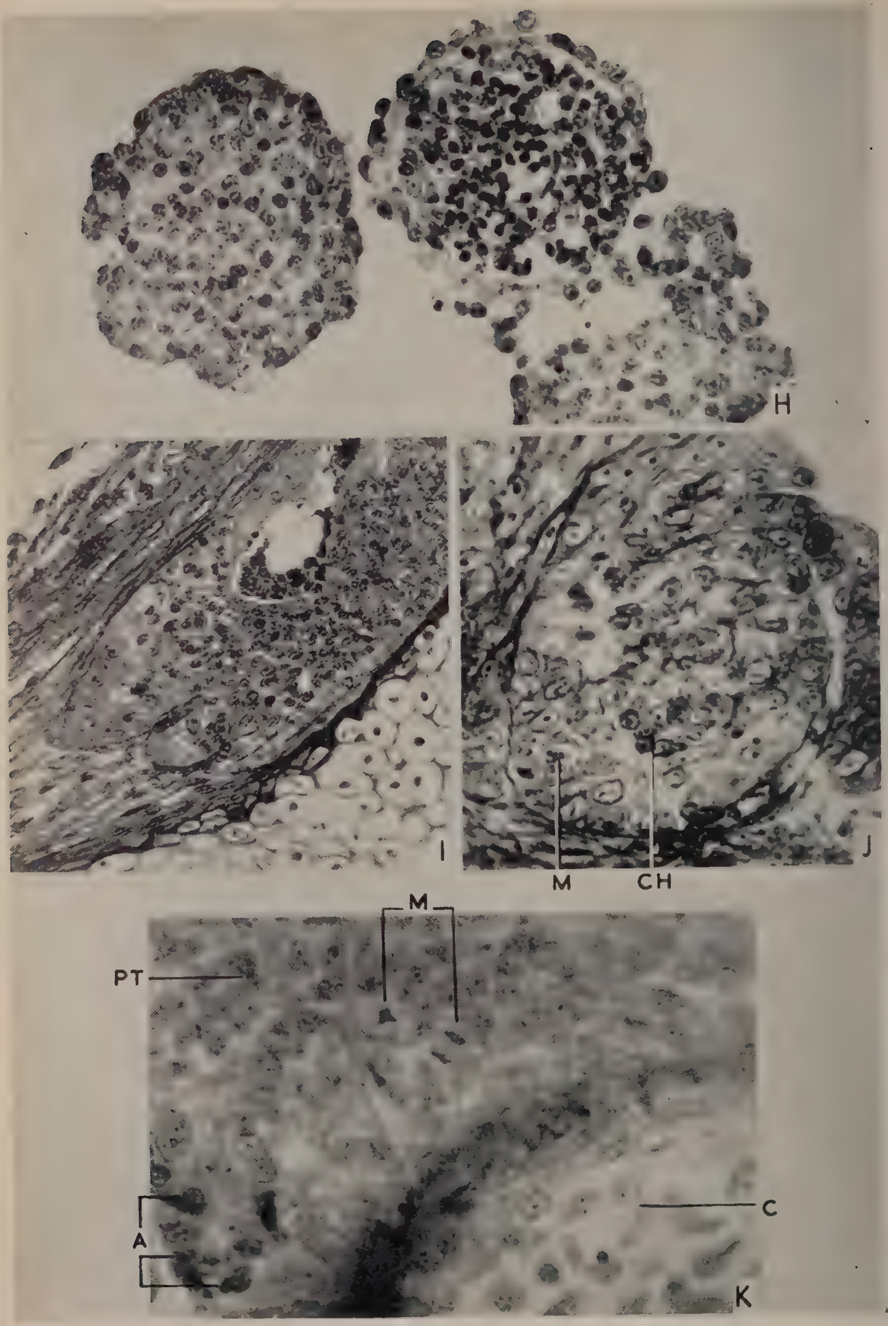

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Plate 2

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